

End of the line for smallpox virus?

An article on page 748 of this issue raises the question whether remaining stocks of smallpox virus should be physically destroyed. That option, chiefly symbolic, should not be followed — just yet.

HERE is an intellectual gift (and puzzle) for the impending holiday season: now that the nucleotide sequence of the smallpox virus is completely known, and the details incorporated in the appropriate databanks, should not the remaining stocks of smallpox virus be destroyed? The case for doing so is simple. Stocks of the virus are potentially a danger, even potential sources of general re-infection of the human population, so long as they continue to exist. So much is clear from the British incident at Birmingham in 1978, when injudicious manipulation of the virus in a university laboratory led to the death of a technician and the subsequent suicide of the academic responsible. In principle, the consequences of the accidental release of smallpox organisms into the general population would now be greater: vaccination against smallpox was abandoned throughout the world in 1985 (most countries had given up the practice much earlier) so that a substantial fraction of all populations now lack immunity.

There is a further argument for destruction. The eradication of smallpox is a bright feather in the cap of World Health Organization (WHO), which set eradication as a goal in 1967 and, with meticulous if costly enthusiasm, set about the attainment of its goal. Who says that international organizations never achieve anything worthwhile? There has been no report of a natural case of smallpox since 1977 (in Somalia). And success has brought two benefits: a disease that as recently as a century ago killed a quarter of a million people a year in Europe alone is no longer a public health problem, and we have a shining example of how it is possible to eliminate infectious diseases altogether, given time, energy and a little luck. (Smallpox was an obvious target because of the high risk that an infected person would be killed, and an attainable one because vaccines are effective prophylactics).

The article now published was stimulated by a meeting in 1986 of a WHO specialist group which recommended that existing stocks of smallpox virus should be destroyed once the nucleotide sequence had been determined. The argument was that techniques even then available would allow the reconstruction of the virus from the known nucleotide sequence. What was true in 1986 is even more true now. So why not fall in with the notion that existing stocks of smallpox virus (at Atlanta and Moscow, the only two repositories of intact virus left) should be destroyed? One reason is general in character. Destroying the existing stocks would be symbolic only. Is there any reason to fear that the Centers for Disease Control at Atlanta and the Institute for Viral

Preparations in Moscow will be less than zealous in safeguarding the material in their freezers?

The arguments against disposal vary in their seriousness, but all turn on the possibility that the human race will be re-infected by smallpox at some stage in the future, when the tools for typing the strains of virus in circulation would be needed urgently. That might happen when corpses, by chance infected with smallpox, are recovered from the permafrost of, say, Siberia and examined in the laboratory. The chances of that happening are slim, but it is technically possible.

The more likely route to reinfection with smallpox or something like it is that one of the existing poxviruses may mutate to acquire the virulence of smallpox. There are several candidates of which the most obvious is the monkeypox virus, which can already infect people as well as monkeys. (The sole host of smallpox virus is the human being, which is one reason why rapid eradication has been possible.) Now that smallpox has disappeared from the list of conditions that diagnostic physicians include among the ailments that may affect their patients, it is likely that reinfection by either route or some other would have spread beyond the person first infected, whereupon there would be a panic. Against that remote possibility, it would be comforting to know that, if necessary, authentic samples of the smallpox virus plaguing recent centuries could be reconstructed quickly from the nucleotide sequence or, more probably, from the clones of sub-sequences of the viral genome that have accumulated and which are now registered with WHO. Before destruction is allowed, should there not be some test of the speed with which the intact virus could be regenerated?

There is also the issue of the route by which classical smallpox evolved its special virulence. To be sure, much may be learned from studies with other poxviruses, but authentic smallpox may be necessary at some stage. Destruction of existing stocks should be undertaken only when that important nugget of understanding has found its way into people's heads, and into the textbooks. But then it should be undertaken gleefully. Destruction then (as now) would be symbolic only, but it would be an important symbol of the eradication of risk from human life. Presumably even those who believe that biodiversity should be maximized would not object. □

■ This double issue contains the complete index for 1993. *Nature* will not appear next week. The next issue will be dated 6 January 1994.



Congress sees instability in Russia as threat to space station deal

Washington. Congressional supporters of the space station being planned by the National Aeronautics and Space Administration (NASA) are becoming increasingly alarmed that, having become reliant on full Russian participation, the station could quickly fall victim to political instability in Russia following the recent elections.

Their strong support is needed to continue US annual funding of \$2 billion for the project. But the Congressmen are angry at the Clinton administration for rushing forward with plans for the Russian collaboration. They feel that the plans are short on detail, and vulnerable to a host of uncertainties, highlighted by the recent Russian election results.

George Brown, chairman of the House of Representatives' Science, Space and Technology Committee and a key space station supporter, claims that both the White House and NASA lack the staff and expertise needed to manage the huge, complex and speedy collaboration now being proposed with the Russians.

"We've moved rather precipitously into this engagement," says Brown. "It's not the engagement, but the depth of analysis that bothers me. We have taken on something which may be beyond the scope of what we understand."

Brown, who has just returned from a visit

to the site at Baikonur in Kazakhstan from which Russian components of the space station would be launched, says that he is in favour of more co-operation with Russia, but wants it to be co-operation which will succeed. "We may be getting into a situation here which is doomed to run into problems," says Brown.

He adds that "any single failure" will now kill the station in the House, which last year voted to continue it by a wafer-thin 216–215 margin. "If Russia falls out the station will go down, just as it will if there is any re-estimate of the costs."

Goldin: under pressure from Congress

Some of those who accompanied Brown to Kazakhstan complain privately that director Yuri Koptev, the director of the Russian Space Agency (RSA), was unable to provide any solid information on how the proposed collaboration would work.

The mission found the launch site virtually deserted. The rocket launch pads were in good repair, but the surrounding infrastructure is in need of "substantial upgrad-

ing", says Brown, estimating that this might cost around \$100 million.

Meanwhile Koptev and Dan Goldin, the administrator of NASA, signed a space co-operation agreement covering collaborative missions to the Russian MIR space station. They hope this will lead to a formal agreement on the international space station proposed last month, to be launched by 12 Russian and 19 US shuttle missions from 1997.

Goldin claimed that the Russian involvement would enhance both the space station itself, and the chances of approval in Congress. "The president is very strongly behind it, as are key members of Congress," he said.

Goldin estimates that the US will probably pay Russia a total of "somewhat less than \$1 billion" for its help with the space station completion, including the \$400 million already promised in advance of station construction and launch. Further details of the station collaboration are due to be worked out at meetings this week between NASA and the RSA.

Asked about the prospects of yet further design changes, Goldin was emphatic that November's design proposal is the final one. "This is it," he said. "We do not want to spend one single minute more on redesign."

Space analysts are optimistic about the space station's funding prospects, largely because of the Clinton administration's firm backing and associated decision to reposition the project as a central pillar of its policy towards Russia (*Nature* 365, 681; 1993).

"At least this has attracted high level White House interest," says John Pike of the Federation of American Scientists. "Clinton has re-established a geopolitical place for the space station." Pike says that some dependence on Russia was inevitable if the Russian contribution was to be great enough to hold down US costs and thus secure congressional support.

Pike observes that if the United States is worried that the space station is just one Russian coup away from a speedy and final demise, then Russia is entitled to point out that just one vote in the US Congress could kill it too. Perhaps that has both parties suitably reassured: it certainly leaves their lesser partners — Japan, Canada and Europe — betting on an interesting double.

But as the Clinton administration, including Admiral Bobby Inman, the new Secretary of Defense, moves hastily to revise its entire foreign policy in the wake of the Russian election results, all bets may soon be off.

Colin Macilwain

IMAGE
UNAVAILABLE
FOR
COPYRIGHT
REASONS

Canadian science gets Nobel gift

Ottawa. Michael Smith, the British-born Canadian chemist who won this year's Nobel prize for his work on site-directed mutagenesis (*Nature* 365, 684; 1993), announced last week that he is to donate half of his Can\$500,000 (US\$380,000) prize-money to support basic research into brain function and in particular the origins of schizophrenia.

The other half of the prize-money will be used to help attract more young people, especially women, to pursue scientific studies, and to create a "better public awareness of science in Canada".

Speaking at a dinner held in his honour by the Medical Research Council (MRC) of Canada, which has supported his research for 28 years, Smith said that he had personal reasons for being interested in schizophrenia. But he added that he was also confident that research on the brain will eventually help solve many human diseases, from manic depression to Parkinson's disease.

The MRC's response to Smith's gift has been to create the Michael Smith Award of

Excellence, consisting of a medal and Can\$50,000, which will be awarded each year for "exceptional creativity and innovation in health research".

The council will support one graduate studentship, one postdoctoral fellowship and one new faculty salary award, totalling more than Can\$775,000, for individuals whose research focuses on the molecular genetics of brain disorders. It will also work with the Schizophrenia Society of Canada and the pharmaceutical industry to establish a research chair on schizophrenia.

The federal Department of Industry has also responded with a Can\$500,000 commitment for the promotion of science, while the British Columbia government has established a Can\$1 million endowment for schizophrenia research and science promotion. For their part, the Schizophrenia Society of Canada and the Canadian Alliance for Research on Schizophrenia have jointly promised to raise a Can\$1 million endowment for research training in schizophrenia.

David Spurgeon

EU states back biotech patent reforms

London. The governments of the 12 member states of the European Union (EU) last week approved a European Commission directive that would standardize their national laws on patenting life. But five years of negotiations may yet come to nothing if, as seems probable, the European Parliament vetoes the entire proposal.

The commission had revised a pro-industry draft, initially produced in 1988, following fierce opposition from the European Parliament. Members of the parliament want stringent conditions on patenting innovations in human, animal and plant genetics, reflecting widespread concern about the social and ethical implications of such techniques.

As a result, the revised directive, approved by EU trade ministers at a meeting in Brussels, acknowledges the concerns of animal welfare groups by forbidding patents on processes for creating transgenic animals — and on the animals themselves — where the suffering or physical handicaps on the animals concerned are “out of proportion to the objective pursued”.

The directive also rules that parts of the human body “as such” (including cells, proteins, genes, and — at the insistence of Italy — “the products of conception”) should not be patented. It accepts, however, that “isolated parts”, including complete genes of known utility, should not be unpatentable simply because of their human origin.

Another major concession would let farmers sow seeds from their own crops on their own fields without paying further royalties. The so-called farmer's privilege has provoked strong feelings, especially in developing countries. Both the Uruguay Round of the General Agreement on Tariffs and Trade and the revised UPOV convention on the protection of plant varieties have left the question open for individual countries to settle as they see fit.

The biotechnology industry unanimously supported the original directive. But it is now divided in its opinion. One school of thought says that no directive is better than the revised directive. For example, a representative of the UK Bioindustry Association says that while the farmer's privilege is appropriate to traditionally bred varieties, it will prevent companies producing transgenic crops from recovering the costs of research and development.

Opponents also say that the directive's original objective of clarifying patent legislation in Europe is not as important as it was five years ago, because subsequent decisions by the European Patent Office (EPO) have shown that the European Patent Convention does not prohibit patents on plants and animals. They are concerned that now the directive will complicate patent decisions by formalizing ethics as a criteria for

patenting, beyond novelty, non-obviousness and usefulness.

But Nigel Pool, head of external regulatory affairs at Zeneca Seeds, says that the details of the directive are secondary to its political importance, and that if it is passed, it will send a strong message of confidence to investors in European biotechnology.

The EU ministers' approval is said to be linked to the publication the week previously of Jacques Delors' white paper (policy document) on competitiveness, growth and unemployment, which identifies biotechnology as one of Europe's three key growth areas.

But animal rights groups and medical charities feel that the directive does not go far enough in restricting patents on either animals or human genes; and the stage is now set for a fierce debate when the revised proposals — once confirmed by the

full Council of Ministers — are returned to the European Parliament for a second reading.

Here feelings run high. Earlier this year, for example, the parliament overwhelmingly passed a resolution calling on the EPO to reverse its decision to issue a patent on Harvard University and Dupont's “Oncomouse”.

Under the Maastricht treaty, final approval of new national legislation will require a single text thrashed out by a conciliation committee, and accepted by majorities in both the Council of Ministers and parliament.

Many observers believe that the strong emotions provoked by animal and gene patents are making the chances of reaching agreement on socially-acceptable patent legislation increasingly remote.

David Dickson and Declan Butler

Reprocessing plant gets green light

London. Almost two-thirds of those who replied to the British government's request for comments about Thorp — the thermal oxide reprocessing plant built by British Nuclear Fuels (BNFL) at Sellafield — said that they were opposed to the plant's operation.

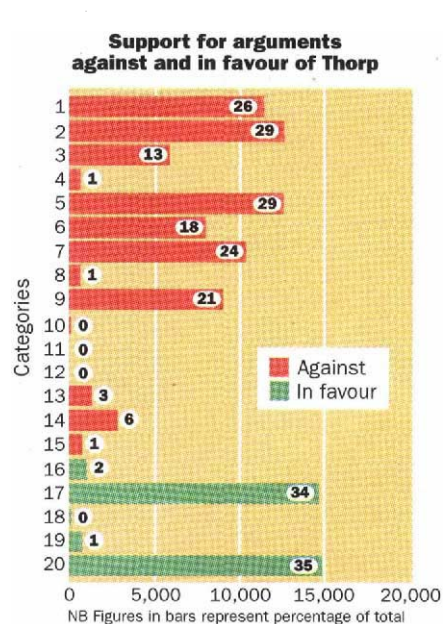
The most popular arguments against starting up the plant were concern about the increase in radioactive discharges into the environment (quoted by 29 per cent of respondents), and demands that no decision should be taken before a new public enquiry (29 per cent), a point raised last week in a letter from almost 30 members of the US Congress to Prime Minister John Major.

Despite such objections, BNFL was given permission to start operation last week, signalling the government's decision to leave the question of whether to reprocess or not up to market forces, as long as the appropriate environmental and non-proliferation criteria are satisfied.

The strongest arguments quoted by respondents in favour of allowing the plant to start up were its commercial benefits, quoted by 35 per cent — BNFL estimates that the reprocessing plant will generate about £2 billion for the British economy — and general comments in favour of reprocessing.

Over 42,500 responses were received during the two month consultation period. Letters were received from Japan, Australia and Ireland as well as the UK, of which a total of 63 per cent were opposed to the operation of the reprocessing plant.

Fiona Gammie



Code: Arguments against Thorp: 1 no justification for reprocessing; 2 calls for public hearing/public inquiry; 3 second consultation; 4 increase in Kr-85 and I-129 discharges; 5 increase in discharges; 6 increase in stockpiles of plutonium; 7 human health effects; 8 assessment methods; 9 discharges from Sellafield adversely affect environment; 10 waste management; 11 current litigation; 12 authorizations/assessments don't comply with legislation; 13 risk/accident during transport of radioactive waste; 14 risk of accident at Sellafield; 15 environmental image of Britain.

Arguments in favour: 16 trading image of Great Britain; 17 pro reprocessing; 18 new authorization; 19 no inquiry; 20 economic benefit.

CERN seeks an extra \$330 million for LHC

Munich. The council of the European Laboratory for Particle Physics (CERN) has confirmed its commitment to the construction of the planned Large Hadron Collider (LHC), and has asked for proposals from CERN management as to how non-member states might contribute towards its financing.

Christopher Llewellyn Smith, who takes over next month as the director-general of CERN, told a meeting of the council in

Geneva last week that the most recent cost estimates, including total costs of experimental areas and experiments, were about SFr2.6 billion (US\$1.7 billion) over the ten-year period 1995–2005.

The machine itself will cost SFr2.23 billion of the total, a price which has been approved as “realistic” by an independent review committee, headed by Robert Aymar of the French Atomic Energy Commission

(CEA). (This committee also approved the technical feasibility of the current design, and concluded that the project “should be approved rapidly.”)

But constructing the LHC at the proposed price and timetable will require an additional contribution of SFr500 million from non-member countries, if contributions of CERN member states are to be kept as promised at their current level. As a result, the council has urged CERN to report back within three months on ways which other countries might become involved.

“Council wishes that such involvement should be on the understanding that usage on a significant scale must involve the provision of resources to suit both CERN and the non-member states concerned,” it said in a statement issued after the meeting.

In pursuit of this goal, Llewellyn Smith will continue to investigate the possibility of reaching cooperative agreements on LHC experiments, particularly with US and Japanese scientists following the abandonment by the United States of the Superconducting Supercollider (SSC).

At the same time, he has been asked to prepare a series of alternative scenarios, and to indicate how the SFr500 million deficit might be accommodated in the absence of international funding, for example by stretching out the 10-year construction timescale, or trimming planned experiments.

In an address to last week’s council meeting, Llewellyn Smith said that LHC would welcome intellectual input from non-member state physicists. But he underlined that participation should be linked with a financial contribution to the project.

Before the demise of the SSC, US high energy particle physicists had open access to CERN facilities such as the Large Electron-Positron Collider (LEP) because approximately equal numbers of physicists from CERN member states were able to work on US-based projects. “Now this equilibrium has completely changed,” says Lyndon Evans, incoming director of the LHC. “US scientists will now be expected to make a contribution to the cost of the LHC if they want to take part in experiments.”

Last week’s council meeting also approved a proposal designed to increase the chances of smaller member states obtaining construction and other contracts, a sore point with countries such as Spain which claim to have been unfairly treated in the past.

The council agreed that, in future, if the lowest bid for a particular contract comes from a country which already enjoys a high level of contracts in proportion to its financial contribution, then CERN will enter into negotiations the two lowest bidders from countries which have “poorly balanced return coefficients” to see if a contract can be agreed with them.

Alison Abbott

Old AIDS news dressed up as new

London. The *Sunday Times* has again attacked both *Nature* and its editor John Maddox for allegedly failing to publicize the views of those who do not believe HIV causes AIDS. In a headline printed over six readers’ letters in its issue of 19 December, the newspaper wrote that “the world’s leading scientific journal” is playing a “sinister game”.

But it did not present any new evidence in support of its own views, nor did it report on scientific research being done on AIDS. It limits its coverage to printing the readers’ letters — four in favour of and two attacking its stance — and to expanding one of these into a short news story.

The only letter from a researcher working on AIDS was from John Moore of the Aaron Diamond Research Center in New York. Moore points out that, rather than ignoring the views of molecular biologist Peter Duesberg that HIV does not cause AIDS, he and other researchers have rejected Duesberg’s arguments as scientifically unsound. He says that the case for HIV has been “proven to our intellectual satisfaction”.

In contrast, Dennis Parke, a retired professor of biochemistry at the University of Surrey, writes in support of the *Sunday Times*’ “exposé”, and puts forward his own theory of AIDS — which includes HIV infection. Parke says that he had to start his own journal to publish “several controversial papers rejected by other journals for their heretical views”.

Parke’s views were repeated in a news story elsewhere in early editions of the newspaper, which repeated his claim to have seen AIDS cases in 1968. But neither the letter nor the news report points out that it is widely recognized in the medical community that AIDS has been around for even longer; two cases from the 1950s have been reported in the clinical literature.

Similarly, the newspaper reports — under the logo “AIDS: why we won’t be silenced” — the discovery of several HIV-negative cases of Kaposi’s sarcoma as if this is a new finding. Again, however, these cases have been reported in the medical



As the *Sunday Times* sees it

research literature for some time.

Three of the remaining four letters accused *Nature* of “scientific censorship” for allegedly failing to report the views of people who do not believe that AIDS is caused by HIV. One gives most prominence to *Nature*’s alleged handling of scientific papers on a different topic. The remaining letter accuses the *Sunday Times* of using its “campaign” on AIDS to increase the circulation of the newspaper.

Other British newspapers have reported on the argument between *Nature* and the *Sunday Times*. Simon Jenkins concluded in the *Times* on Saturday (18 December) that AIDS researchers (and others) should declare who funds their research, clearly unaware that published scientific papers invariably carry this information.

In a different vein, the *Guardian* wrote in its issue of 12 December that, while many agree that AIDS is not spreading through the heterosexual population at the rate which was predicted six years ago, “it is the *Sunday Times* alleged lack of balance which has alienated the establishment”.

In reply to the *Guardian* article, Andrew Neil, editor of the *Sunday Times*, said that the idea of there being some doubt about the role of HIV in causing AIDS had been suppressed “in most of the media with all the rigour of a one-party state”.

Neil also said that the *Sunday Times* had found it difficult to print letters critical of what he calls the papers “revisionist stance” because they contain “abuse rather than reasoned argument”. Elsewhere in this issue (page 716) we reprint one of the letters the newspaper refused to publish earlier this year.

Maxine Clarke

Survey battle leads to plagiarism verdict

Hong Kong and Tokyo. A bitter dispute between two epidemiologists at the University of Hong Kong has, after a court battle lasting seven years and costing over HK\$16 million (US\$2.1 million), resulted in the world's first legal verdict in which a scientist has been found guilty of plagiarism.

In August, an appeals court in the British colony confirmed the verdict of Hong Kong's High Court in April last year that Lam Tai Hing, a reader in the university's department of community medicine, had made unauthorized use of a questionnaire devised by a lecturer in the same department. An internal inquiry is now underway in the university to determine if Lam should be dismissed.

But the verdict has split the world of epidemiology. Some researchers welcome the way it has focused attention on the value of questionnaires as research tools, and the amount of original effort that can go into their compilation. Others claim that the extent of the plagiarism was relatively limited, and that the court ruling sets a dangerous precedent for the future progress of epidemiology. Some even describe it as a "miscarriage of justice".

The conflict began in January 1980, when the then head of the university's community medicine department, Michael Coulbourne, introduced Linda Koo, a new lecturer in the department, to his collaborator John Ho Hung Chiu, honorary professor of radiation oncology at the university.

Coulbourne encouraged the two to continue research which he and Ho had been carrying out into the unusually high incidence of lung cancer in non-smoking Chinese women. At the end of the same year, Coulbourne, having retired from the department, encouraged Lam Tai Hing, another lecturer then on a postgraduate course in Britain, to take up the same line of research.

Over a period of 20 months, Koo and Ho drafted 14 versions of a questionnaire, which in its final form included 69 main questions, and numerous subquestions. They then carried out fieldwork, which was completed in April 1983.

Lam returned to Hong Kong in October 1981. Supported by the new head of the department, J. W. L. Kleevans, he obtained a separate grant, and began work on his own questionnaire, which passed through four stages of drafting.

At this point, claims Koo, amicable relations in the department began to deteriorate. Lam and Kleevans approached Ho to join Lam's project, but Ho declined. Koo alleges she was subsequently harassed and victimized by Kleevans and Lam; Kleevans admitted in court that he had suggested she leave the department on at least three occasions.

In 1986, Koo says she began to suspect that Lam had used parts of her questionnaire

to construct his own. He admitted having a copy of her questionnaire, but denied using it in his own work, and claimed that she had given it to him.

Koo and Ho appealed to the university vice-chancellor. But attempts at mediation failed, and one month later the two researchers issued a writ against Lam, charging infringement of copyright and misuse of confidential information.

The subsequent court case focused on the degree of similarity between the two questionnaires, including both the final and draft versions — making comparison a difficult and time-consuming process — and on whether epidemiological researchers usually share such information or keep it confidential.



Koo: victor in court case

ally share such information or keep it confidential.

One witness called on Koo's behalf was Robert MacLennan, senior principal research fellow with the Queensland Institute of Medical Research in Australia, and a pioneer of research into the incidence of lung cancer in non-

smoking women.

MacLennan pointed out apparent similarities in some of the questions and their sequence in the two sets of questionnaires, and argued that the likelihood of Lam creating his independently was "extremely low", a view that was backed up by a British linguistics expert. He also argued that few investigators are willing to share their questionnaires at an early stage in their research.

But Richard Peto, director of the Cancer Studies Unit at the University of Oxford in Britain, a world-respected authority on links between smoking and cancer, argued in Lam's defence that there was very little similarity between the questionnaires.

Peto claimed that it was normal practice for epidemiology researchers to share information about their questionnaires, and argues that these should not be subject to copyright protection.

Whatever the merits of such arguments, Lam's case was weakened by the fact that, after six years of denial, he eventually admitted that he may have made some limited use of Koo and Ho's questionnaire in formulating his own. The court subsequently ruled that he had infringed Koo and Ho's copyright, and misused confidential information that he had obtained "surreptitiously".

The appeal court upheld this verdict, but stated that the word "surreptitiously" did not mean that Lam "stole" the question-

naire. Rather, the court ruled that he had come into possession of a document which he knew was confidential and was not entitled to use.

Peto feels strongly that the courts' findings are a miscarriage of justice. "If people were to put the two [final] questionnaires side-by-side, they would see that there has been no impropriety," he says. But the transcript of the hearing shows that the court was persuaded by "substantial" similarities between earlier versions of the two questionnaires.

Suketami Tominaga, director of the Aichi Cancer Center Research Institute, who says that Japanese epidemiologists usually share questionnaires, even in the early stages of research, suggests that competing epidemiologists "should get permission" to use elements from a questionnaire compiled by another researcher.

Takeshi Hirayama, formerly of the National Cancer Institute in Tokyo, whose studies in the 1960s were among the first to link passive smoking to lung cancer, says that the important factor to emerge from the case is the originality of questionnaires. "Success or failure of a study depends on the quality of the questionnaire," he says.

Hirayama says he supports Peto and MacLennan, and that he is saddened by the court case. He describes Lam as a "good researcher", and Koo as a driving force in the argument that diet is an important co-factor in lung cancer in non-smoking women.

H. K. Ma, the dean of the medical faculty of the University of Hong Kong, says the university's internal inquiry will try to determine the "degree and seriousness" of Lam's plagiarism, describing him as "one of the faculties most respected academics".

Koo's charges of harassment and victimization have yet to be thoroughly investigated. These have been made on numerous occasions, including in a letter to the university's vice-chancellor in January 1987, when Ho and Koo complained about the actions of Kleevans, including restricting Koo's access to department facilities, blocking her application for a grant, and trying to persuade her to give up research on lung cancer.

But the university did not investigate the charge at the time because, it claims, the whole issue had been made *sub judice* by the court case, adding that, as Kleevans left the university in 1987, "there would seem to be little point in pursuing that issue now".

Koo feels she is being victimized as a whistleblower, and points out that while Lam has been promoted during the trial to reader, she has remained a lecturer for 13 years. Ma says she is unaware of the 1987 complaints, but has investigated more recent charges made by Koo against the present head, and has found "no grounds at all" for complaint.

David Swinbanks

AIDS, HIV and the *Sunday Times*

■ This letter was submitted for publication to the *Sunday Times* on 23 March this year, in response to an article published on 21 March. The newspaper declined to publish it. Professor Anderson is now in the Department of Zoology, University of Oxford.

SIR — Your article entitled "Epidemic of AIDS in Africa 'a tragic myth'" gives a seriously misleading impression of the magnitude of the AIDS problem. You again give credence to the claims of Professor Peter Duesberg that HIV is not the cause of the disease AIDS. Last month, two carefully controlled studies of homosexual and heterosexual men in San Francisco (*Nature* 11 March 1993) and homosexual men in Vancouver (*Lancet* 13 March 1993) were published demonstrating that only those with evidence of HIV infection developed AIDS, and that in those infected with HIV, drug use is no more frequent in those who develop AIDS than in those who do not. Yet again, Duesberg's claims that drug use rather than HIV is a cause of the disease AIDS have been firmly rejected.

More broadly, your article appears to present two lines of argument. First that the high prevalences of HIV observed in various parts of Africa do not reflect a major AIDS epidemic; and second, that the high prevalences reported in the medical and scientific literature are false and unrepresentative of the true picture. Your correspondent draws these conclusions on the basis of a 2-month study by a television presenter, Joan Shenton. He fails to mention the many years of study by experts and highly qualified scientists and epidemiologists, whose work has been peer-reviewed and published in leading scientific and medical journals, that totally contradicts the non-expert opinion of Shenton. The evidence charting the emergence and rapid growth of the AIDS epidemic in Africa (and other regions of the developing world) over the past 12 years is detailed in hundreds of scientific publications. In the worst-afflicted countries in Africa the prevalence of HIV infection in the sexually active population has risen to 20–40% in some urban centres. As a direct consequence of the immunodeficiency caused by HIV infection, the prevalences of other killer diseases such as tuberculosis have also been rising in many regions. Persistent tuberculosis infection in HIV-infected individuals also acts to spread tuberculosis to those not infected with HIV.

Your correspondent cites a Dr H. Bialy who states that most HIV-positive tests are false in Africa because of the presence of malaria in many patients. In 1985 an association between malaria and the likelihood of getting a positive HIV test result was reported. However, much subsequent work with the current generation of highly reliable HIV tests (virtually 100% specific) has found no association between present or past infection with malaria and the likelihood of getting a positive HIV test result. He also cites Professor Beverly Griffin, who reports very low levels of HIV infection (1–2%) in Malawi, for further evidence of limited HIV spread in Africa. Griffin's results are based on small samples of 5–8-year-old children from rural regions. Current evidence from Africa suggests that 35% of infants born to infected mothers acquire HIV. Infected children have a short life expectancy in developing countries (a conservative estimate is 4 years from birth) and hence a 1–2% prevalence of HIV in 5–8-year-olds implies a 14–28% prevalence of HIV in pregnant women a number of years ago. Malawi is not a country from which data on the prevalence of HIV are readily available, but a study in 1990 in an urban setting of 6,482 pregnant women revealed a 22.8% prevalence of HIV infection. In much of Africa, the urban centres are the worst hit at present, but infection is steadily spreading into the rural areas.

Your article seriously undermines the efforts of many concerned scientists and medical personnel to persuade governments in Africa, and international aid agencies, of the urgency of trying to slow the spread of HIV. In some urban centres in Africa, AIDS is already the leading cause of adult mortality (for example Abidjan, Cote d'Ivoire) and in the coming decade this trend is likely to be repeated in many other regions of the world. The research on the spread of AIDS and the mortality included by the epidemic in Africa is published in leading journals such as the *Lancet*, the *British Medical Journal*, *Science* and *Nature*. The basis of the views presented by the supposed experts cited in your article are less easy to validate. Much of the article relies on the views of H. Bialy, who is associated with the journal *Bio/technology*. While *Bio/technology* is a reputable journal in the field of biotechnology, it is a sister journal to *Nature* only in the sense that the *Sun* is a sister journal to the *Sunday Times*. A search for papers published by H. Bialy in 1992 revealed one on transgenic wheat and another on pathway analysis in plant cells. If the *Sunday Times*'s views about AIDS are to be seen as anything more than sensationalist, it should choose its "experts" more carefully.

Geoff Garnett
Roy Anderson
Department of Biology,
Imperial College,
London SW7 2BB, UK

Calendar reform

SIR — Jews reckon time from the biblical creation of the world (set at 3761 BC); the Romans from the founding of Rome; and the Moslems since the Hegira (AD 622). In AD 526, the Emperor Justinian introduced the current system of reckoning time from the birth of Christ, set at 753 AUC (*ab urbe condita*, "since the founding of Rome") by the monk Dionysius Exiguus.

In the BC/AD system that Justinian introduced, the numbering of years is ordinal, not cardinal; there is no year zero; and the numbers increase in opposite directions (whereas time flows in the same direction). As a result, time intervals across the BC/AD boundary cannot be calculated algebraically — the time interval between 1.5 BC and AD 1.5 is one year, not three years. As well as being inconvenient to those who deal with history and ancient human events, the BC/AD way of reckoning years singles out an event — the birth of Christ — that has no significance to many civilizations.

I propose that the beginning of calendrical time could be set at the beginning of the current Julian cycle (12.00 noon Greenwich mean time, 4713 BC), established in 1582 by Joseph Scaliger and still used by astronomers. A constant — 4,713 years — would then have to be added to the AD dates and the BC dates would have to be subtracted from 4714 (the Scaliger year equivalent to AD 1). (To simplify the arithmetic, a round unit such as 10,000 years could be added to the AD dates instead.)

Setting the birth of Christ at 25 December of the year 10,000 from the beginning of what could be appropriately called the "human era", would make the year AD 1 into the year 10,001 and the year 1 BC into the year 10,000. All BC dates would thus be subtracted from 10,001.

Setting the beginning of the human era at 10,000 BC would date the first year of Scaliger's Julian period at the year 5288; the beginning of the Egyptian calendar (4241 BC) at the year 5760; the founding of Rome at 9248, the birth of Christ at 10,000, the fall of the Roman Empire at 10,476, the French Revolution at 11,789 and the present year (1993) at 11,993. I suggest that the new calendar is adopted in the year 2000 (new year 12,000).

Cesare Emiliani

Department of Geological Sciences,
University of Miami,
Coral Gables,
Florida 33124, USA

Correction: FGIN

■ A letter in Correspondence about the Fidia Georgetown Institute for the Neurosciences (*Nature* 366, 399; 1993) should have included among the signatories the following: Pier Franco Spani (Chairman, Institute of Pharmacology, University of Brescia). □

Recipe for a good research laboratory

A Japanese laboratory illustrates the benefits of autonomy (and the responsibility that goes with it) and budgetary predictability in creating a framework for productive research.

Sagamihara. To celebrate the beginning of the next millennium, UNESCO or some such body might think of awarding a prize to the best laboratory in the world. What could be a better celebration of a century's remarkable achievement and a more powerful reinforcement of still timorous hopes for the future than such a recognition of well ordered and imaginative research? (If UNESCO thinks well of the idea, it had better get moving quickly; there is not much time left.) But the chances are that the prize would even then go to the Institute for Space and Astronautical Science (ISAS) at this site West of Tokyo, which is contiguous with Metropolitan Tokyo even if it is confusingly in a different prefecture.

ISAS has significantly interesting roots. It owes its existence to the early post-Sputnik enthusiasm of academics at the University of Tokyo for space research of almost any kind. (The first experiment, organized by the Department of Industrial Technology, was the *horizontal* launch of a rocket 23 cm long to confirm ideas about drag in the lower atmosphere.) By 1964, a group of academics from the physics department and the engineering faculty had pooled resources to build and launch first sounding rockets, then satellites.

Ten years ago, their successors moved from a scruffy building in Tokyo proper to splendidly functional buildings on this site, which was previously a US military base and which, by legend, was one at which Japanese researchers were within an ace of developing a surface-to-air missile (based on infrared guidance) when bombs fell on Hiroshima and Nagasaki in 1946. What follows are notes compiled during a three-day visit (for a different purpose) last week which may throw some light on why some laboratories are better than others.

The constitution helps. ISAS was created as a research institute within the University of Tokyo, which has until recently been the Japanese way of supporting some university research well while neglecting the rest of it. That means that the institute has from the outset been supported exclusively by the Ministry of Education. Its senior members were invariably members of the Tokyo faculty; now there is a sprinkling of people from the universities of Nagoya, Osaka and Kyoto.

The most striking mark of the institute's success is that, in the past 20 years, it has launched 22 Earth satellites entirely with its own resources and, during that long period, has had only one failure (in the late 1970s).

The present goal is to launch one satellite a year, either in January or July when down-range restrictions in the interests of Japan's vociferous fishermen are suspended, but there were two in 1985 in preparation for the rendezvous the following year with Halley's comet. What rocket-launching organization would not give its eye-teeth for such a record of reliability?

That numerical index does not fully reflect the academic benefits of this predictability. But it is now, for example, institute policy to launch one X-ray satellite every four to six years. The argument is that, having encouraged the growth of an X-ray astronomy community in Japan (nearly 100 people, two-thirds of them students), it is only proper to cater for its interests with fresh data and for its aspirations by designing ever more advanced telescopes.

Ingenuity, just sheer neatness, is another characteristic of what goes on here. ISAS, for example, has been the most diligent of space research organizations in the exploitation of the momentum a spacecraft can pick up through a close encounter with some planetary object to win extra lifting power (mass into a low orbit) from the rockets available. As early as 1990, a satellite called *Hiten* was put into an eccentric orbit around the Earth in such a way that, after eight successive close encounters with the Moon, it occupied an eccentric orbit reaching out nearly three times as far as the radius of the lunar orbit.

More recently, the satellite called *Geotail*, launched in 1992 by a US rocket from NASA's Kennedy Space Center in Florida, is now making the most detailed observations yet of the solar wind in the complicated wake created by the Earth's motion around the Sun. *Geotail*, already further away than *Hiten* in its eccentric orbit about the Earth, is still alive.

But ingenuity is everywhere. There is, for example, a complicated bundle of wires that can be wound on what looks like a large fishing-reel that will, by means of moveable hinges, turn into a rigid boom when it is unwound. Six such devices will be needed for the 8-m radiotelescope now being planned for launching in 1996. That will be the world's first dedicated radiotelescope in orbit, and the first serious use of a space component for Very Long Baseline Interferometry (VLBI). Perhaps the people who gave the rest of us *origami* are best equipped to fold an 8-m telescope into an instrument bay only 2.4 m in diameter.

The engineers seem confident that it will work. They can, after all, point to the success of the extendible optical bench it was necessary to use to fit four parallel X-ray telescopes into the fourth X-ray satellite *Asca*, launched in February this year. In the event, the collapsed version of the structure did extend itself to give the telescopes the planned focal length of 3.5 m.

Patience is another of ISAS's virtues. Thus the engineers use telemetry data from rockets during their launch phase not simply to check that they are still intact, but also to improve the performance of their successors. The result is that the lifting power of the rockets now being used (solid-fuelled three-stage rockets whose diameter has been restricted to 1.2 m) has been increased three-fold over the years. People seem confident that the new rocket (twice as wide, but not much longer) being developed (and to be used first for launching the radiotelescope) will similarly be improved.

Why does all this work so well? There are several explanations. First, there is a separation of functions between ISAS and the more recently created National Space Development Agency (NASDA), whose remit is exclusively the development and launching of applications satellites (for communications, for example). Among other things, that means that space science is not cut back when applications work runs over budget.

Second, ISAS gains extra autonomy from being comprehensively in charge of everything it does, from engineering design to operations.

Third, engineering is as much honoured as science. In the project teams constituted afresh for each satellite launch (usually five years in advance), engineers and scientists work as equal partners. To rub the point in, directors-general are chosen alternately from the two sides of the church. Graduate students (about 100 in total) are expected to muck in with the rest, and may well find themselves responsible for executing some mid-orbit course correction from the modest control room.

The other strength of ISAS is its human scale. The present staff is fewer than 300 (who ensure that the 100 graduate students keep their noses to the grindstone). Senior people may find themselves carrying responsibility for two or three separate functions, in flat contradiction of what the management schools forever preach. Inevitably, official working hours are an unattainable dream. It could only happen in Japan.

John Maddox

Zircon can take the heat

Joel D. Blum

THE remarkable properties of zircon (ZrSiO_4) have allowed geochronologists to unravel further details of the Cretaceous/Tertiary (K/T) boundary impact event, which coincided 65 million years ago with the extinction of over half the species on the Earth. Zircon is a mineral unrivalled in its resistance to thermal, chemical and mechanical breakdown and has long been exploited as a resilient carrier of radioactive uranium, allowing researchers to look through complex geological histories and date the primary ages of crystalline rocks. Now zircon has been put to the ultimate test. On page 731 of this issue, Krogh *et al.*¹ demonstrate that tiny crystals of zircon (1–4 millionths of a gram), blasted from the Chicxulub crater in Mexico by an extraterrestrial impact equivalent in energy to about 10,000 times the world's nuclear arsenal², preserve an isotopic record of both the age of the target rocks and the age of the impact event itself.

Krogh *et al.*, in this and a previous study³, provide geologists with an important new tool for directly linking impact craters to specific layers in sedimentary rocks that contain a record of the geochemical and biological trauma induced by the impact events. For impacts that are geologically young, tiny glass spherules derived from rock melted by impact and blasted into the atmosphere (microtektites) are sometimes found in marine sediments and provide a time marker as well as a geochemical link back to the source crater. For the giant impact that occurred 65 million years ago, these glassy spherules have been found in K/T sediments at only two sites — both of which were within about 1,000 km of the crater. The glasses have a distinct neodymium and strontium isotopic signature that has allowed them to be linked directly to melts found within the Chicxulub impact structure^{4–6}. At other locations on Earth, however, K/T boundary glass has been chemically transformed to clay, obscuring the chemical fingerprint of the materials.

Crystals

Krogh *et al.*^{1,3} have established that zircon crystals present in target rocks can survive the impact as well as subsequent chemical alteration and provide a unique isotopic fingerprint of the target material even in the absence of impact glass. Zircon contains an important isotopic record because, on crystallization, the mineral incorporates uranium into its crystal structure but excludes lead. Following crystallization, two independent radioactive clocks begin ticking at different rates: ^{238}U decays to ^{206}Pb

with a half-life of 4.5 billion years, and ^{235}U decays to ^{207}Pb with a half-life of 0.7 billion years. Some of the zircons analysed from the K/T boundary have essentially remained closed chemical systems and thus the two U–Pb clocks yield the same (or concordant) ages. Others, which display shock-induced planar deformation features (like the zircon on the cover) and granular textures, lost some of their radiogenic Pb and thus yielded two different (or discordant) ages. Krogh *et al.*¹ used a routine method in U–Pb geochronology which combines the two parent–daughter systems on a 'concordia diagram' (see their Fig. 1) and allows estimation of both the primary age (upper intercept) and the age of disturbance (lower intercept) for a population of zircons. There was nothing routine, however, about obtaining precise lead isotope ratio measurements on single zircons weighing 1–4 micrograms. Samples contained only 5–200 picograms of radiogenic lead, and therefore extreme care had to be exercised in an ultraclean laboratory to reduce background lead contamination to just ± 1 pg.

The most notable finding of Krogh and co-workers was that most single zircon crystals from Chicxulub crater breccias, as well as K/T boundary sediments from both Haiti and Colorado, fell on a single U–Pb isotopic variation trend, indicating a target basement age of 545 ± 5 million years followed by episodic Pb loss about 55–65 million years ago. This story is complicated slightly by a subset of samples that fell on a second trend, indicating that the target also had some material with a basement age of about 420 million years.

Krogh *et al.*'s results strengthen three conclusions that were proposed by previous workers on the basis of strong (but somewhat circumstantial) evidence: first, that the Chicxulub structure is indeed the source of K/T boundary ejecta, and the buried crater at Manson, Iowa, was not involved; second, that ejected material found in the marine Haitian K/T sediments was derived from the same source as ejecta from the continental Colorado K/T sediments; and third, that Palaeozoic silicate basement rocks beneath ~2 km of sedimentary rocks were ejected from the Chicxulub crater during the impact.

Thirteen years have passed since Alvarez *et al.*⁷ proposed, on the basis of rather sparse geochemical evidence, that an asteroid struck the Earth at the end of the Cretaceous period, wreaking havoc on the environment. In the ensuing years, investigators working to test this theory have documented a wealth of evidence in its support, leaving no viable alternative

theory. Debate is now focusing more specifically on whether the impact directly caused the K/T boundary extinctions⁸. Before the identification of the site of the impact, several extinction mechanisms were proposed that could apply to any impact, regardless of the target location. These included global cooling and darkness from the dust lofted by the impact⁷, and acidic rainfall resulting from nitric acid produced by shock heating of the atmosphere⁹. Now that Krogh and co-workers' study and previous work^{4,10–12} have firmly established that the Chicxulub structure was formed by the K/T impact, researchers are moving on to investigate environmental consequences specific to the target geology.

Climate

In an unlikely coincidence, the K/T bolide landed in an area (the Yucatán Peninsula) that is underlain by a thick sequence of volatile-rich rocks including limestone (CaCO_3), dolomite (MgCO_3) and anhydrite (CaSO_4). An inevitable outcome of the impact, therefore, was the degassing of at least some of these rocks by melting and shock-devolatilization, dramatically changing the chemistry of the Earth's atmosphere. The huge (but difficult to quantify) amounts of CO_2 and SO_2 added to the atmosphere would have had an effect much like that which humans are currently inflicting on it by burning fossil fuels. Predicted consequences of the impact would be global cooling and acid rainfall for several years as a result of increased SO_2 levels^{13,14}, followed by global warming for tens of thousands of years as a result of increased CO_2 concentrations^{15,16}. Recognizing this, scientists now have the unique opportunity to study how the Earth's biogeochemical cycles respond to sudden changes in atmospheric composition, and whether (or how) this contributed to the nearly simultaneous extinction of over half the species on the Earth. □

Joel D. Blum is in the Department of Earth Sciences, Dartmouth College, Hanover, New Hampshire 03755, USA.

1. Krogh, T. E. *et al.* *Nature* **366**, 731–734 (1993).
2. Alvarez, L. W. *Physics Today*, 24–33 (July 1987).
3. Krogh, T. E., Kamo, S. L. & Bohor, B. F. *Earth planet. Sci. Lett.* **119**, 425–429 (1993).
4. Blum, J. D. *et al.* *Nature* **364**, 325–327 (1993).
5. Sigurdsson, H. *et al.* *Nature* **353**, 839–842 (1991).
6. Premo, W. R. & Izett, G. A. *Meteoritics* **27**, 413 (1992).
7. Alvarez, L. W., Alvarez, W., Asaro, F. & Michel, H. V. *Science* **208**, 1095–1108 (1980).
8. Johnson, K. R. *Nature* **366**, 511–512 (1993).
9. Prinn, R. G. & Fegley, B. *Earth planet. Sci. Lett.* **83**, 1–15 (1987).
10. Hildebrand, A. R. *et al.* *Geology* **19**, 867–871 (1991).
11. Swisher, C. C. *et al.* *Science* **257**, 954–958 (1992).
12. Sharpton, V. L. *et al.* *Nature* **359**, 819–821 (1992).
13. Sigurdsson, H., D'Hondt, S. D. & Carey, S. *Earth planet. Sci. Lett.* **109**, 543–559 (1992).
14. Brett, R. *Geochim. cosmochim. Acta* **56**, 3603 (1992).
15. Blum, J. D. & Chamberlain, C. P. *Science* **257**, 1104–1107 (1992).
16. O'Keefe, J. D. & Ahrens, T. J. *Nature* **338**, 247–249 (1989).

Anchoring glycine receptors

Stanley C. Froehner

WORKING out how synapses are formed has occupied neurobiologists for many years. On page 745 of this issue¹ Kirsch *et al.* now report a major step in understanding how membrane proteins are reorganized during this process. They show that a peripheral membrane protein, termed 'gephyrin' (from the Greek for 'bridge'), is essential for glycine receptor clustering at inhibitory synapses.

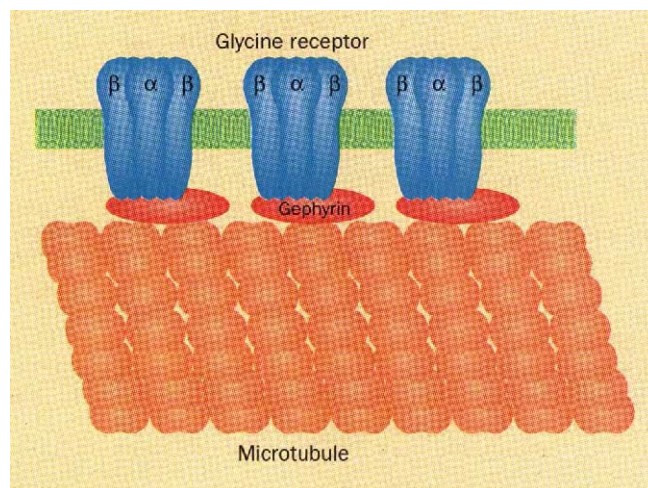
Consider the complexity of synapse formation in the brain. A single neuron often receives synaptic input from thousands of presynaptic axons. And exquisite coordination between the pre- and postsynaptic cells is required to achieve proper alignment of the presynaptic terminals with postsynaptic sites, which possess high densities of the correct neurotransmitter receptor, thus ensuring rapid and specific synaptic transmission. The neuromuscular junction, where extremely high concentrations of nicotinic acetylcholine receptors are confined to the muscle synaptic membrane, has attracted particular attention in the expectation that principles established at this synapse will be applicable to the central nervous system. Kirsch *et al.*, in contrast, took the direct approach by studying the glycinergic synapse in the spinal cord.

Glycine receptors mediate synaptic inhibition in the spinal cord by gating chloride ions². Like other members of the ligand-gated ion channel family, the glycine receptor is a pentameric oligomer composed of two subunits, the ligand-binding α -subunit and a structural β -subunit. Both subunits span the membrane several times and together make up the core of the functional complex. A third protein, gephyrin (formerly known as the 93K protein) is closely associated with the glycine receptor core and co-purifies with it. Gephyrin is a peripheral membrane protein associated with the cytoplasmic side of the postsynaptic membrane. This location, and the tendency of gephyrin to form large aggregates, prompted the suggestion that it might serve as an anchor and immobilize glycine receptors at the synapse.

As a direct test of this idea, Kirsch *et al.* used antisense oligonucleotides to deplete neurons of gephyrin. Rat spinal-cord neurons in culture express glycine receptors, which are initially localized intracellularly. After a few days, the

receptors appear on the cell surface, and become organized into small clusters (thought to be sites of synaptic contact) on the cell body and along the neurites. Gephyrin aggregates appear before the receptor clusters, but eventually the two become colocalized.

Kirsch *et al.* treated neuronal cultures with an antisense oligonucleotide spanning the start codon of the gephyrin messenger RNA and found that expression of gephyrin protein was decreased by more than 95 per cent. Concomitantly, the



A model for the clustering of glycine receptors in postsynaptic membranes. Glycine receptors are pentamers composed of two different subunits, α and β . Gephyrin, a peripheral membrane protein, links the receptors to microtubules and anchors them in place.

number of cells with glycine receptor clusters, and the number of clusters in each cell, were dramatically reduced. The effect was reversible and specific (there were no alterations in cell morphology or in expression of glycine receptors, and sense oligonucleotides had no effect). Gephyrin, then, is essential for the formation of glycine receptor clusters.

How it does this is not known, but gephyrin's ability to interact with the cytoskeleton is likely to be important. Several lines of evidence show that it binds polymerized tubulin³. The interaction between gephyrin and tubulin is a high-affinity, cooperative one, which approaches a stoichiometry of 1:4 at saturation. So gephyrin may provide a link between glycine receptors and the subsynaptic tubulin, thereby immobilizing receptors into clusters within the postsynaptic membrane.

How general is such a mechanism likely to be? Synaptic receptor clustering has been most thoroughly studied at the neuromuscular junction, where basal lamina proteins such as agrin, which are

derived in part from the innervating neuron, direct clustering of nicotinic acetylcholine receptors in the postsynaptic membrane. Gephyrin is not present at this synapse, but another peripheral membrane protein, the 43K protein, is closely associated with cytoplasmic domains of the acetylcholine receptor and may subserve a similar role (for review see ref. 4). Recombinant 43K protein forms membrane-associated clusters when expressed in non-muscle cells, and acetylcholine receptors become associated with 43K protein clusters when the two proteins are coexpressed. Association between the 43K protein and actin has been proposed as a mechanism for immobilizing acetylcholine receptors at the neuromuscular synapse. So although the proteins involved are different (gephyrin and the 43K protein share no similarities in amino-acid sequence), the general concept that a peripheral membrane protein links neurotransmitter receptors to the submembrane cytoskeleton may apply at both synapses.

Gephyrin could be involved with other neurotransmitter receptors. It is expressed widely in the nervous system, even in some regions of the brain which lack glycine receptor α -subunits⁵. In this respect, the existence of several forms of gephyrin generated by alternative splicing of RNA transcripts may provide some hints⁶. Selective use of four different cassettes, encoding sequences of 14, 16, 26 and 36 amino

acids, could produce a large number of functionally diverse proteins. The cassettes are restricted to the amino-terminal half of the protein, raising the possibility that this variable part associates with different membrane receptors, depending on the cassettes used, while the constant carboxy-terminal region of the protein binds microtubules. As the biochemical characterization of other ligand-gated synaptic receptors advances, it will be interesting to learn if they associate with particular isoforms of gephyrin, or whether each receptor type has its own unique peripheral membrane protein. □

Stanley C. Froehner is in the Department of Physiology, University of North Carolina, Chapel Hill, North Carolina 27599, USA.

1. Kirsch, J., Wolters, I., Triller, A. & Betz, H. *Nature* **366**, 745–748 (1993).
2. Langosch, D., Becker, C.-M. & Betz, H. *Eur. J. Biochem.* **194**, 1–8 (1990).
3. Kirsch, J. *et al.* *J. Biol. Chem.* **266**, 22242–22245 (1991).
4. Froehner, S. C. A. *Rev. Neurosci.* **16**, 347–368 (1993).
5. Kirsch, J., Malosio, M.-L., Wolters, I. & Betz, H. *Eur. J. Neurosci.* **5**, 1109–1117 (1993).
6. Prior, P. *et al.* *Neuron* **8**, 1161–1170 (1992).

Brown fat and yellow mice

Jeffrey M. Friedman

GENERATIONS of physiologists have tried to get to grips with the complex networks that control body weight and fat content. Help was needed and, as evidenced by work reported on page 740 of this issue¹ and elsewhere^{2,3}, molecular biology is providing it. Lowell *et al.*¹ engineered transgenic mice in which brown fat was ablated and which in consequence became obese; and in earlier work (discussed in News and Views⁴ in April, but in a different context) Bultman *et al.*² and Miller *et al.*³ identified the causal gene for a dominantly inherited form of obesity, the yellow agouti (*A^y*) mutation.

It has long been thought that the amount of adipose tissue in an individual is physiologically controlled⁵. This must be largely true, as the body weight of most people seldom changes radically (unless they're starving, dieting or ill). This hypothesis has also been supported by physiological, clinical and genetic experiments, the simplest interpretation of which is that a complex homeostatic system (energy homeostasis) regulates caloric intake and energy output to maintain a constant body weight. But despite attempts to identify the components of this system, little is known about the contributing genetic elements. The hypothalamus seems to be an important regulator because specific lesions in it have been shown to perturb the nutritional state in animals⁶. And administration of several peptides into the central nervous system can alter food intake⁷.

Nonetheless, mysteries abound. How tight is the control of body weight? How does the periphery communicate information concerning nutritional state to the CNS? What effector systems maintain constancy of body weight?

Into the fray come several groups. Lowell *et al.*¹ among them. They tested the role of brown fat in energy homeostasis by using a toxic transgene to ablate brown fat. The transgene fuses the brown-fat-specific uncoupling protein promoter to a diphtheria toxin-coding sequence. In all cases, the brown fat in the transgenic mice apparently disappeared; the mice also put on weight, and had all the hallmarks of obesity, including increased body fat and severe insulin resistance.

Brown fat is a heat-generating organ, found principally in neonatal animals and humans in the intrascapular region⁸, and is present in much smaller amounts than white fat. Unlike white fat it expresses an unusual protein known as uncoupling protein, and contains many mitochondria which give it its brown colour.

Uncoupling protein is a mitochondrial proton channel that is not coupled to

oxidative phosphorylation⁸. So when a proton gradient is established across the inner mitochondrial membrane, activation of the uncoupling protein leads to the uncoupled passage of protons through the channel and the generation of heat. Expression and activation of uncoupling proteins is usually mediated by the sympathetic nervous system and is directly controlled by norepinephrine. This mechanism is part of the adaptive response to cold temperatures. That brown fat is involved in regulating body weight was first suggested by the observation that over-fed animals show increased brown fat thermogenesis⁹. Nevertheless, although uncoupling protein is clearly involved in thermoregulation, its role in the regulation of nutritional state was less certain.

Now, however, Lowell *et al.* show that there is a dynamic interplay between the periphery and the CNS in the control of energy homeostasis. They identify brown fat as an important component of the system in rodents, although the precise means by which it acts to restrain body weight is yet to be fully established.

Decreased energy expenditure by brown fat is characteristic of a variety of obesity syndromes, including genetic obesity and obesity secondary to chemical or physical lesions of the CNS¹⁰. This decreased activity had been assumed to be secondary to a general decrease in the activity of the sympathetic nervous system. However the demonstration that ablation of brown fat leads to obesity would seem to indicate that brown fat activity, and perhaps energy expenditure in general, may be more directly involved in determining body adiposity.

Does the level of energy expenditure correlate with a predisposition to obesity? And are alterations in energy expenditure causal in the development of obesity? Preobese infants and adults followed longitudinally, as well as some reduced obese people, expend less energy than lean control subjects^{11,12}. This difference in energy expenditure, coupled with differences in food intake, results in an obese phenotype which, when fully developed, is associated with apparently normal levels of energy expenditure and food intake (when indexed to lean body mass). From the data of Lowell *et al.* it seems that modulation of energy expenditure by itself may play a primary role in the development of obesity. In retrospect, this conclusion is supported by the apparently salutary effects of exercise, as well as of uncouplers of oxidative phosphorylation such as dinitrophenol, on body weight.

There is much more to clarify, however. For instance it is not easy to calculate

whether the amount of calories normally expended by brown fat fully accounts for the increased amount of white fat seen in the transgenic animals. Clearly brown fat cannot simply be an effector organ; if it were, the transgenic animals would be expected to reduce their food intake to match the reduced energy output. Moreover, the amount of brown fat decreases dramatically after weaning (although it does not disappear in adults), meaning that perhaps there is a critical period in development when subtle perturbations in energy homeostasis have an effect on adult adipose mass. The use of transgenic mice expressing toxin genes which can be activated at different times during development may help to address this question; the answer should bear on the old notion that overeating or a decrease in activity in children may contribute to obesity later in life.

The only caveat concerns the vagaries of transgenic mice. It is difficult to prove that the only lesion in the transgenic mice is in brown fat. The uncoupling protein promoter is responsive to norepinephrine and could be expressed at a low level in the hypothalamus or elsewhere, the normal gross appearance of the CNS in the transgenic animals notwithstanding.

The obesity in Lowell and colleagues' transgenic mice is not the first example of a novel combination of a promoter and coding sequence leading to obesity. Nature first accomplished this task thousands of years ago in the yellow agouti mouse — *A^y* mice first turned up in ancient China as curiosities because of their brilliant yellow colour and associated obesity. It has turned out that *A^y* is a true dominant mutation, also associated with increased skeletal growth and a predisposition to several cancers¹³.

The cloning of the yellow agouti gene, assisted by a convenient rearrangement on mouse chromosome 2, has shown that the phenotype of *A^y* mice is the result of a subtle inversion that places a new promoter upstream of the agouti gene². This leads to the ubiquitous expression of the agouti gene product, which is normally restricted to skin³. The agouti protein, as predicted by previous studies using skin transplants, has the features of a secreted

1. Lowell, B. B. *et al.* *Nature* **366**, 740–742 (1993).
2. Bultman, S. J., Michaud, E. J. & Woychik, R. P. *Cell* **71**, 1195–1204 (1992).
3. Miller, M. W. *et al.* *Genes Dev.* **7**, 454–467 (1993).
4. Jackson, I. J. *Nature* **362**, 587–588 (1993).
5. Kennedy, G. C. J. *Physiol. Lond.* **145**, 578–592 (1952).
6. Keesey, R. E. in *Obesity* (ed. Stunkard, A.) 144–166 (Saunders, Philadelphia, 1980).
7. Friedman, J. M. & Leibel, R. L. *Cell* **69**, 217–220 (1992).
8. Nicholls, D. G. & Locke, R. M. *Physiol. Rev.* **64**, 1–64 (1984).
9. Rothwell, N. J. & Stock, M. J. *Nature* **281**, 31–35 (1979).
10. Bray, G. A. & York, D. A. *Physiol. Rev.* **59**, 719–809 (1979).
11. Bogardus, C. *New Engl. J. Med.* **318**, 467–472 (1988).
12. Roberts, S. B., Savage, J., Coward, W. A., Chew, B. & Lucas, A. *New Engl. J. Med.* **318**, 461–466 (1988).
13. Wolff, G. L., Roberts, D. W. & Galbraith, D. B. *J. Hered.* **77**, 151–158 (1986).

protein. Sites of synthesis of the dysregulated version include fat, brain, liver and almost every other tissue. So agouti protein is one which can, when expressed in the wrong place and at the wrong time, cause obesity. The precise locus and mode of action of dysregulated agouti remains to be determined.

Between them the new studies raise as many questions as they answer. But

obesity is a serious health issue, and the advent of the molecular era of research on the topic gives cause for optimism that those questions can be tackled — and even answered. □

Jeffrey M. Friedman is in the HHMI Research Laboratories, Rockefeller University, 1230 York Avenue, New York, New York 10021, USA.

ELECTRON MICROSCOPY

The ultimate analysis

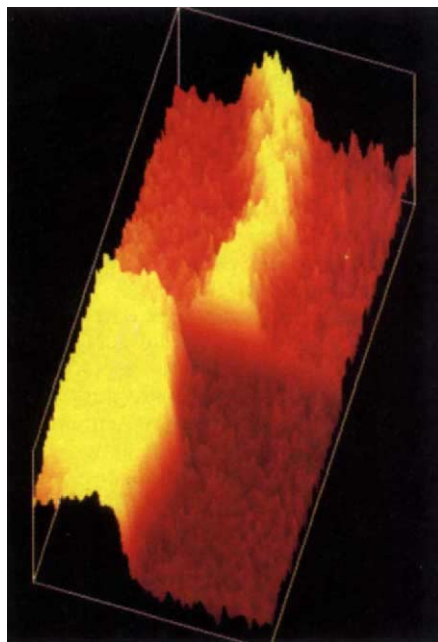
L. M. Brown

THE ultimate reductionist goal of the scientist engaged in unravelling the properties of solids is to locate and identify the component atoms, one by one; then to determine the possible electronic transitions which the electrons of those atoms can undergo. With the announcement by three groups — two elsewhere in this issue^{1,2}, and one in an earlier paper³ — of atomic column-by-column imaging, accompanied by electron energy-loss spectrometry from a specified column, this goal has come within reach of many laboratories around the world. 'Columns' here means stacks of atoms, like grapefruit piled high in the supermarket.

The chain of evolution of the new techniques is ramified. The first single-atom images, accompanied by spectroscopy, were made many years ago by E. W. Muller; a modern review by Miller⁴ tells the story. In this technique, the atomic mass of each atom can be established as the atoms are extracted from the specimen. But the electronic structure of the embedded atom is not accessible. Many years later, Crewe⁵ managed to produce images of single atoms in a scanning transmission electron microscope incorporating a field emission electron gun to achieve enough current in his atomic-sized probe. His work demonstrated that the best way of getting the required contrast was to look at electrons scattered to large angles, in the 'annular dark-field detector'. Crewe's method was aimed at biological problems, where the atoms are generally not in crystalline arrays. But it rendered visible atoms embedded in their environment, and because it was a scanning technique, it opened the door, in principle, to the acquisition of electron energy-loss spectra from individual atoms. However, when it was applied to crystals, strong specular Bragg reflections confused the images.

It was Howie's suggestion⁶ that much larger angles of collection should be used, so that only incoherent scattering contributed to the image. Armed with this technique, Pennycook and Boatner⁷ were able five years ago to demonstrate inco-

herent imaging of atomic columns in thin films: a notable advance, because by conventional non-scanning methods the fringes of atomic spacing produced by coherent interference of Bragg beams depend treacherously upon foil thickness, defocusing the microscope, and the pre-



Interface between diamond film and silicon substrate (Muller *et al.*¹). Top, bright band is threefold-coordinated amorphous carbon at the interface; bottom, bright region is fourfold-coordinated diamond film.

cise orientation of the atomic columns: delicate calculations are required to decide if atoms should be white or black!

While these developments were in train, considerable effort was put into improving electron spectrometers. Ideas were borrowed from the efficient β -particle spectrometers used by atomic physicists. Progress owes much to the advent of charge-coupled devices which permit digital acquisition of many channels of a spectrum at once, all under computer control and allowing fast numerical processing.

The two strands of development come together in the ability to image columns of a few tens of atoms, which show as light blobs in the dark-field image; then confidently to put the electron probe at the top of the column, directed down it, and to acquire a spectrum which indicates essentially the density of empty states available to the atomic electrons in the column, giving both the chemical species and the valence state, as well as vital information on the local coordination of neighbours. In this way, Batson² is able to see the different oxidation states of silicon in the neighbourhood of the interface with its oxides, and Muller *et al.*¹ to see atomic interlayers between a silicon substrate and a diamond film grown by chemical vapour deposition. These are applications of great importance, because they allow for the first time a robust view of the chemistry of the nucleation of thin films, on the scale of the initial nucleus.

What can we expect of the new technique? One limitation, as with all electron microscopy, is radiation damage: it will not be possible to study the interfaces between enzymes and their biological reaction products, nor any aliphatic compounds. The best spectral resolution achieved by Batson is 70 meV, so the infrared part of the spectrum is poorly covered. In fact, the uncertainty principle limits the spatial resolution obtainable at such low energy losses, so that only for losses of about 100 eV or higher does the technique really make sense.

On the other hand, the spectra obtainable at these energies are comparable to or superior to those from synchrotron sources, with the bonus of atomic spatial resolution. The cost of a machine to do this is about £400,000, which enables any well-equipped laboratory to carry out the experiments, and certainly compares very favourably with the cost of a synchrotron. So we must expect that in the world of inorganic thin films, where interfaces control the structure and properties of the product, these new techniques will be widely taken up and have a profound impact. In metals and ceramics, where grain boundaries act as chemically active interfaces, the techniques open up a whole new field of experimental grain-boundary chemistry which should lead at last to control and eradication, at the atomic level, of the chief causes of brittleness and failure of materials in service. □

L. M. Brown is in the Cavendish Laboratory, Madingley Road, Cambridge CB3 0HE, UK.

1. Muller, D. A., Tzou, Y., Raj, R. & Silcox, J. *Nature* **366**, 725–727 (1993).
2. Batson, P. E. *Nature* **366**, 727–728 (1993).
3. Browning, N. D. *et al.* *Nature* **366**, 143 (1993).
4. Miller, M. K. *Int. Mater. Rev.* **32**, 221–240 (1987).
5. Crewe, A. V. *Electron Microscopy in Material Science* (ed. Valdre, U.) 163–207 (Academic, New York, 1971).
6. Treacy, M. M. J., Howie, A. & Pennycook, S. J. *Inst. Phys. Conf. Series*, No. 52 (ed. Mulvey, T.) 261 (1980).
7. Pennycook, S. J. & Boatner, L. A. *Nature* **336**, 565 (1988).

Missing mismatch repair

Miroslav Radman and Robert Wagner

Two decades of research with bacterial systems have revealed a highly sophisticated enzymatic DNA-editing system that controls the precision of DNA replication and recombination through the detection of unpaired and mispaired bases in DNA (see ref. 1 for review). Predictions that a malfunction of this repair system in humans should accelerate carcinogenesis have now proved to be correct — papers in *Cell*²⁻⁴ describe the discovery of a gene that is apparently involved in mismatch repair and which, when mutant, predisposes its bearer to several common cancers.

Mutations in this gene, called *HNPCC* (for hereditary non-polyposis colon cancer) or human *MSH2* (for *MutS* homologue 2), occur in about 0.5 per cent of the population and apparently increase dramatically the risk of colon, ovarian, uterine and kidney cancers. The gene shares considerable homology with the bacterial *mutS* and yeast *MSH2* genes, which are both involved in the earliest stages of mismatch repair. Indeed, previous work with bacteria and yeast made the search for the human *MSH2* gene serendipitously straightforward; one group² isolated it by using primers in the polymerase chain reaction that had been employed in the identification of *mutS* homologues in yeast.

The idea that HNPCC might be caused by defective mismatch repair came from findings that HNPCC tumour cells show a characteristic size instability in simple sequence repeats (the 'accordion effect')^{2,3}. These sequence blocks, most often di- or trinucleotide repeats, are notoriously 'slippery' for the DNA replication machinery such that unpaired bases are occasionally left in the parental or newly synthesized strand. These small deletions or insertions are normally rectified by the mismatch repair system. Although the origin of simple sequence repeat blocks has yet to be explained, it may be that they are 'scars' left from the repair of broken chromosomes when recombinational repair could not operate. Repair may have involved the addition of such sequences to the broken ends of chromosomes, followed by annealing to rejoin the fragments.

Perhaps the strongest evidence that the mutation responsible for HNPCC affects mismatch repair comes from the finding that extracts of HNPCC tumour cells are deficient in such repair *in vitro*⁴. However, mismatch repair levels in HNPCC germ-line cells heterozygous for a mutation in the human *MSH2* gene were indistinguishable from those in normal cells. The authors propose that some regulatory

event may inactivate the functional human *MSH2* gene in tumour cells^{2,3}, but it seems as likely that a second mutation may inactivate the functional copy of the gene. A second *MSH2* mutation was found in one HNPCC tumour (the system used may not have allowed detection of every *MSH2* mutation). Such mutational inactivation may, in fact, be an obligate step in HNPCC tumorigenesis.

The predisposition to cancer associated with the HNPCC syndrome may be the result of a greatly increased frequency of specific oncogenic mutations caused by a generalized mutator effect in homozygous *MSH2* mutant cells. But the apparent absence of homozygous *MSH2* mutations in the germ-line cells of HNPCC patients may indicate that such homozygosity, and the attendant high level of mutation, is lethal. If so, survival of somatic cells homozygous for *MSH2* mutations may require some specific oncogenic alteration. This would explain the tissue specificity of HNPCC tumours, because not every oncogenic alteration may allow cell survival. Perhaps the relatively good prognosis for patients with HNPCC colon tumours (and sporadic tumours with an HNPCC phenotype) reflects the relatively low fitness of such tumour cells resulting from the mutator phenotype. The deleterious effect of such a potent mutator may also result in the strong selection for *MSH2* revertants or suppressors during the later stages of tumour progression, or during the establishment of tumour cell lines, so that the extent of mismatch repair deficiency in human carcinogenesis may be underestimated.

HNPCC tumours seem to be chromosomally more stable than other types of tumours, although bacterial *mutS* mutants exhibit considerable chromosomal instability as a result of recombination between diverged repeats. But bacteria appear to have only one *MutS* function, whereas in eukaryotes *MSH* has different roles, so *MSH2* may be primarily a replication editor and *MSH3* a recombination editor. Perhaps some common cancers and cancer-prone syndromes with hyper-recombination phenotypes involve *MSH3* mutations. □

Miroslav Radman and Robert Wagner are in the Laboratory of Mutagenesis, Institute Jacques Monod, CNRS/University Paris 7, 2 Place Jussieu, Tour 43, 75251 Paris Cedex 05, France.

1. Radman, M. & Wagner, R. *Chromosoma* **102**, 369–373 (1993).

2. Fishel, R. *et al.* *Cell* **75**, 1027–1038 (1993).

3. Leach, F. S. *et al.* *Cell* **75**, 1215–1225 (1993).

4. Parsons, R. *et al.* *Cell* **75**, 1227–1236 (1993).

Geolubrication

WHEREVER two continental plates are moving against each other, earthquakes are a major worry. The plate edges bind together, so that stress slowly builds up at their interface. When the stressed joint finally fails, the two edges suddenly shear past each other, releasing enough energy to devastate the land above. The edges then bind together once more and the process starts again, in the most dramatic example of stick-slip friction known to science.

So Daedalus wants to lubricate the interface. The plate edges would then glide smoothly past each other, dissipating their energy slowly and undramatically. Lubricating oil would be useless at the temperatures and pressures around 100 km down, where the plates clash. A malleable solid such as glass or salt would be a better high-pressure lubricant for the hot rocks. But even the best drilling methods can hardly penetrate 10 km into the Earth. How to get the lubricant down there?

Daedalus's bold plan is to lubricate the fault lines of the Earth with high-level radioactive waste, and to let it melt its way down to the fault. All over the world, large amounts of high-level waste are sitting in water-cooled tanks, awaiting the day when it is cool enough to bury. Bury the waste now, says Daedalus, and bury it in boreholes immediately above the more threatening of the seismogenic faults of the world. Many nuclear power plants have been thoughtfully sited in these earthquake zones already, simplifying the transport problems. Once in place at the bottom of a borehole, the waste will heat up the surrounding rock, and melt its way downwards. Ultimately it will reach the fault, where it will raise the local temperature by hundreds of degrees. The shear strength of rock drops dramatically with temperature, so the waste will create a local weak spot in the stressed fault. A line of boreholes would create many such weak spots. Instead of generating huge and damaging earthquakes at long intervals, the fault would grumble its way through many small, harmless slips, as each weak spot relaxed its own local stress.

Environmentalists should welcome the scheme. At one stroke it gets rid of a dangerous material and reduces the threat of earthquakes. But timing would be tricky. Lubricating a highly stressed fault would simply precipitate an earthquake. You would have to wait until an earthquake had just occurred, and then lubricate the momentarily relaxed fault. Stress would no longer build up in it, and the lethal succession of earthquakes would cease.

David Jones

Is NASA an expensive failure?

Thomas Gold

The debacle of the US space programme is the consequence of the insecure, publicity-seeking policies of the National Aeronautics and Space Administration.

THE succession of NASA failures cannot be ignored. The enormous expense of shuttle launches has removed NASA from competitiveness in the international market for the launch of utilitarian satellites such as those for studying the weather, for international communications systems, or for surface mapping of the globe. At the start of the shuttle programme, NASA announced that the huge investment would be quickly repaid, because it would make space launches enormously cheaper than disposable boosters could ever make it. Now, 20 years on, the opposite is true: the cost per pound launched into a near-Earth orbit is many times greater than that of the unmanned, disposable boosters which have meanwhile been developed in several other countries. Further, disasters and near-disasters make clear that the shuttle is not a secure launch system.

Beyond that, we have witnessed a succession of failures of gigantic proportions. The Hubble space telescope, a \$2 billion enterprise, had a design fault so gross that it could have been detected before launch at relatively small extra expense with quite simple, high-precision measuring instruments. Whether the recent repair mission will turn out to be successful remains to be seen. But its cost (between \$630 million and \$1.2 billion) must temper enthusiasm for a repair that, at best, cannot bring the instrument to the originally expected performance. The number of independent, serious faults that had to be fixed does not make for a good prognosis.

The Galileo mission to explore Jupiter and its satellites, costing more than \$1 billion, may still yield some results, but a mechanical failure in unfurling the spacecraft's antenna will prevent it from signalling all its results back to Earth.

Now, after a costly series of failed shuttle launches, another major project costing nearly \$1 billion, the Mars Orbiter, has been inexplicably lost. Similarly, an Earth-mapping satellite, a continuation of the Landsat series, is now floating uselessly in some unknown Earth orbit. Is this succession of failures to be expected from a programme that has been properly planned, in view of the enormous cost?

The problems of planning space research programmes were discussed with great care and in detail in the early 1970s. Committees of external advisers, some set up by NASA and some by the Office of Science and Technology in the White House, made numerous detailed recom-

mendations which in general favoured a cautious, step-by-step approach to provide a learning period before any major expenditures. The advice of these committees was largely against the design of the shuttle but in favour of improving expendable, unmanned boosters. NASA was advised to practice sending small spacecraft with gradually advancing complexity of instrumentation to the planets, and initially a much smaller space telescope into orbit. Having gained experience from these, larger missions could be undertaken later.

NASA was invariably strongly opposed to such a policy. Its aim, so it seemed to me in the 1970s, from the perspective both of the White House space science panel and the Lunar and Planetary Missions Board of NASA, was to gain approval for the largest project in each field that could possibly be proposed. For a launch vehicle, nothing short of a huge manned shuttle would do, even if the launch would often be for only a small utility satellite. The experience with the Apollo mission to the Moon had given NASA confidence in mega-projects. Such projects, once approved, would be hard to cancel, whereas a succession of mini-projects could always be cut back. I suppose the reasoning was that huge projects would provide the financial stability which this giant, post-Apollo organization now required. The conflict with the external advisory groups was severe. Eventually, both the Nixon White House and the NASA administration disbanded them.

Within the NASA organization, external research funding was given preferentially to a few groups "with whom it could work", meaning those that went along with its policies. This policy was apparently introduced at the top level. For example, a memo (unearthed under the Freedom of Information Act) from George Low, deputy administrator, to James Fletcher, administrator, discusses one proposal: "... incidentally it is a good one. ... I had no objection to his testimony before Congress, but he should realize that being funded by the government and by NASA is a privilege and that it would make little sense for us to fund him as long as his views are what they now are. ...". The memo has a handwritten comment in the margin, apparently by Fletcher, stating "great work".

Many other academic research groups took the view that they would not be able

to change the NASA policies, so they might as well do the best they could by going along with them. NASA tended to represent this attitude in Congress as an endorsement of its plans.

Many of the best scientists and engineers left NASA in the 1970s, some, I have no doubt, because of their disagreement with these policies. Proposals for future missions were selected according to their ability to attract large funds from Congress, and their scientific or utilitarian value was only a distant second criterion. NASA spent large sums for propaganda for its proposals, and is still doing so. It inevitably has enormous support from science-fiction buffs and space enthusiasts, and that support spills over into Congress. NASA nurtured that support with the suggestion that all the current manned space endeavours are the necessary prelude to manned exploration of the planets, commencing with Mars. But a Mars expedition, based on the only propulsion technique now available, chemical rockets, would require a 2-year round trip, which would be extremely hazardous and expensive. If a new and much faster means of propulsion were to be devised, there would be plenty of time then for the preliminary work. Manned space-flight exercises now will do nothing to advance the time when a manned Mars flight becomes a real possibility. I am sure NASA is aware of this, but it prefers to lead the public to believe that a great new era of manned planetary flight is beginning, and that it is laying the groundwork.

Is it possible that this institutional corruption has become so pervasive that NASA can no longer hold together a body of scientists and engineers of the calibre required for their ambitious plans? Are we seeing now a debacle that stems from a mental attitude, rather than from a statistical fluctuation in the rate of accidental failures? If this is so, how must we then view the next gargantuan NASA plan of a permanent space station? This plan involves the immediate commitment of billions of dollars, as well as an open-ended commitment to further support. Of course, it also requires the long-term commitment, once the station is built, of keeping it manned, supplied, functional and safe — if that were possible. □

Thomas Gold is professor emeritus at Cornell University, Ithaca, New York 14853, USA.

Physics in brief

N. David Mermin

What Makes Nature Tick? By Roger G. Newton. *Harvard University Press*: 1993. Pp. 257. \$27.95, £22.25.

IN only a little more than 200 pages and with only an occasional equation, Roger Newton talks engagingly to his readers about Laplacian determinism, vectors, logarithms, differential calculus, time's arrow, Carnot cycles, thermodynamics, entropy, light, relativity, quantum theory, gauge fields and strings, waves, solitons, tachyons, elementary particles, bosons and fermions, phase transitions, superfluidity, superconductivity, symmetry, conservation laws, and many other things. If that sounds like a lot, that's because it is. Two questions come to mind: to whom is such a book addressed, and how well does it manage to convey a sense of the most important developments in physics from Isaac Newton to the frontiers of contemporary research?

According to its preface, the book grew out of the author's talks to a group of colleagues meeting to discuss their various disciplines, and it is intended for non-scientists who know very little mathematics. Roger Newton is thus addressing readers ignorant of technical physics, but at home with both abstract ideas and those aspects of contemporary physics that count as common knowledge among the educated. He is never patronizing, giving his readers a brisk, readable prose. They are clearly expected to catch on fast. You could not use this book as a text in an undergraduate course of the 'physics for

poets' variety, but I can imagine recommending it to a suitably warned non-scientist colleague as a fine way to get a quick and literate glimpse of the things that preoccupy physicists these days.

I say suitably warned because the clarity of the many sketches is uneven. In view of the vast scope this is hardly surprising. When subtle and intricate matters are characterized in a page or two there is a high risk that essential points may end up in the wrong place or be omitted altogether. This can be corrected in the give and take of a discussion group, but it can blemish a printed text. Sometimes omissions are unavoidable, but the author of such a book should hold fast to one rule: readers should always know when they are being told to take something on faith and when they are expected to figure it out for themselves. In the latter case the author has a moral obligation to give them the wherewithal to do it. Newton is not always true to this principle.

In the handful of pages on special relativity, for example, he tries to deduce the length contraction from what is known 'in the trade' as the invariance of the interval — a starting point that is algebraically simple to state, but entirely un-intuitive. Although I believe he wants the reader to accept this as a postulate, he says nothing to forestall a conscientious reader from worrying why it is not evident. He

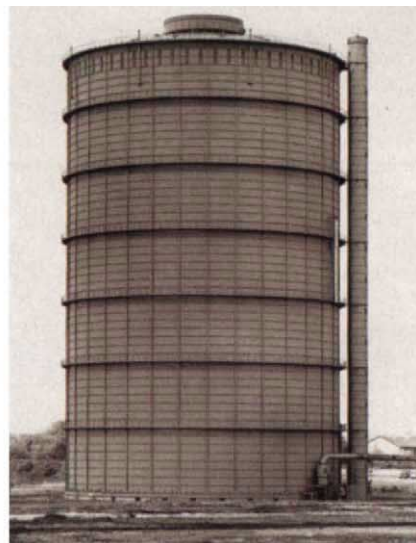
then compounds the confusion by extracting length contraction from the invariance of the interval by an argument that can make sense only to a reader who understands the relativity of simultaneity, which he has yet to mention. When he does come to it (on the next page) he remarks that readers may find it shocking. But they had to understand it implicitly to make any sense of the earlier argument.

This is an example of a pedagogical lapse that could have been repaired. In other cases I suspect the concepts are so subtle and the treatment so sketchy that the sense of puzzlement the reader will surely acquire cannot be relieved by a simple reorganization of the argument.

So in recommending the book to a friend I would urge her to think hard, line-by-line, about what she was reading, but I would warn her that when some hard thought failed to clarify an obscure point, she should not assume that the fault was hers.

"Newton's book is, quite simply, a masterpiece," exudes Sheldon Glashow on the dust jacket in a noteworthy example of the hype that (almost) launched ten thousand GeV, "I wish that I had written it". A masterpiece it isn't — not even a *tour de force*. But I respect Newton's accomplishment. Those few parts of it I could have written I like to think I would have done better, but most of it I couldn't have written at all. This is a noble but flawed attempt to do what nobody else has come close to doing. I hope it will inspire somebody to do even better. □

David Mermin is at the Laboratory of Atomic and Solid State Physics, Cornell University, 532A Clark Hall, Ithaca, New York 14853, USA.



WHAT a gas — three types of gas tanks (or gasometers). The oldest is the multiple section type (left). It works on the same principle as a bell jar upside down in a tank of water. Spherical gas tanks store

liquefied gas under pressure. The piston type (right) is a large, fixed cylinder in which a piston disk floats on the gas. From *Gas Tanks* by B. and H. Becher. MIT Press, £49.50, \$55.

Mapping sp^2 and sp^3 states of carbon at sub-nanometre spatial resolution

David A. Muller*, Yujiun Tzou†, Rishi Raj‡
& John Silcox‡

* Physics Department, † Department of Materials Science and Engineering, ‡ School of Applied and Engineering Physics, Cornell University, Ithaca, New York 14853, USA

THE potential for diverse applications of diamond¹ has been enhanced by the discovery of the chemical vapour deposition process^{2,3} for film formation. The growth of hetero-epitaxial diamond films on silicon is a particularly attractive goal, but only polycrystalline films have so far been prepared in this way⁴. Because of the large lattice mismatch, thin intermediate layers (interlayers⁵) are formed between the diamond and silicon phases, which may contain crystalline SiC (refs 6, 7) or amorphous compounds (SiC, carbon⁸ and SiO₂). An understanding of how diamond nucleates^{5,9–11}, and the role of these interlayers, requires a detailed knowledge of the nature of carbon bonding (sp^2 or sp^3) at the interface. Here we report the use of transmission electron energy-loss spectroscopy (EELS) to obtain a map of sp^2 and sp^3 carbon at a spatial resolution of less than a nanometre across the silicon–diamond interface. We find that diamond nucleates on an amorphous carbon layer, with the transition from sp^2 to sp^3 carbon occurring over less than one nanometre.

A STEM equipped with an electron energy spectrometer can select a specific energy loss, and scan the focused 0.3-nm-wide electron probe over the sample¹² thereby mapping the locations at which that loss occurs. Our observations are tuned to the carbon K-edge (that is, excitations from the carbon 1s orbital to states above the Fermi level). From Fermi's golden rule and dipole selection rules, the observed intensity is proportional to the local density of states (LDOS) with p -like symmetry in the conduction band^{13,14}. Figure 1 shows the background-stripped¹⁵ carbon K-edge loss spectra taken from the diamond and a-C (amorphous carbon) labelled regions of the STEM bright-field image shown in Fig. 2 and recorded in spot mode. This diamond-film specimen was prepared by microwave decomposition of a methane–hydrogen (1/99) mixture, deposited first for 1 h at 640 °C, then 1 h at 750 °C and thinned as a cross-section sample⁹. In a molecular-orbital picture, carbon in the diamond structure has four unoccupied σ^* orbitals, whereas in graphite or sp^2 a-C there are three σ^* and one π^* states separated by

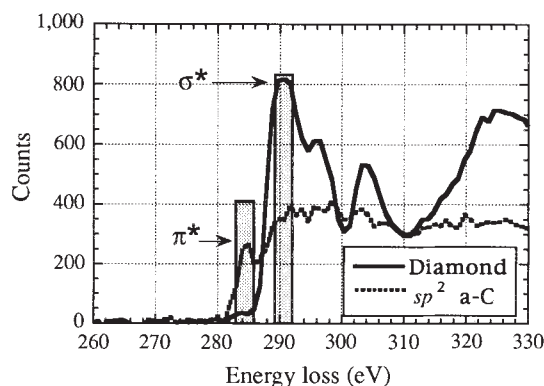


FIG. 1 Carbon K-edge, after background subtraction, taken from the CVD diamond and amorphous carbon layers shown in Fig. 2. The boxes placed over the π^* and σ^* peaks at 285 and 290 eV respectively represent the energy windows used to record the spatial maps shown in Fig. 3.

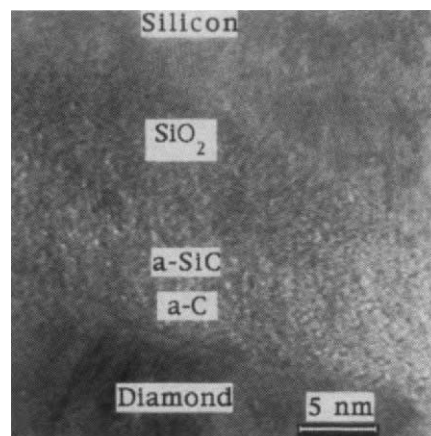


FIG. 2 Bright field STEM image of the CVD diamond-silicon interface recorded with a 10 mrad objective aperture and 10 mrad collection angle, giving a probe size of 0.3 nm and a beam current of 0.12 nA. The electron beam is transmitted through the specimen, normal to the plane of the image and parallel to the buried interface which is viewed in projection. In this 30-nm-thick specimen, ~98% of the atoms will be sufficiently far from the surfaces to have coordination like that of atoms in the bulk. These are the same conditions used for the energy-filtered imaging. The various regions have been identified using EELS (see Fig. 4).

~5 eV. Previous work has identified these peaks by comparisons of the carbon K-edge of known samples of the materials^{16,17} with band structure calculations¹⁸ and by angle-dependent scattering in graphite¹⁹. A feature characteristic of graphite but not a-C is a sharp peak at the onset of the σ^* band. The absence of this peak in the interface region suggests that if microcrystallites of graphite are present, they must be smaller than 1.5 nm (calculations using the XANES computer program show that cluster sizes of ~80 carbon atoms are needed before the LDOS of a graphite cluster resembles that of the bulk material²⁰).

To ensure the high mechanical and electrical stability needed to obtain maps of details of the core loss spectra, the Vacuum Generators HB501A 100 kV STEM was located in a shielded room²¹. The spatial drift is $<0.05 \text{ nm min}^{-1}$ and the energy drift is $<0.06 \text{ eV min}^{-1}$. The EELS signal is recorded digitally in an electron counting mode with single electron sensitivity and a detection quantum efficiency (DQE) of ~90% (ref. 22). This stability and high detector efficiency meant that a slit width (resolution) of 2 eV was sufficient to allow the π^* state (285 eV) and the σ^* state (290 eV) to be separated in spatial maps at sub-nanometre resolution. At least one pre-edge spectrum must also be recorded to give a background which can then be subtracted. The annular dark field (ADF)^{12,23} signal was recorded simultaneously with the EELS and used to check the spatial alignment of the different energy-loss images.

The chemical bonding maps shown in Fig. 3 should be compared with the area of the interface between the silicon and diamond shown in Fig. 2. An amorphous area (width ~10 nm) seen between the two crystalline regions in Fig. 2 contains the high-intensity area seen in the energy-filtered high spatial resolution map obtained from the π^* (285 eV) peak (see Fig. 1) shown in Fig. 3a. The corresponding map for the σ^* (290 eV) peak (see Fig. 1), is shown in Fig. 3b and the spatial distribution of the sp^3 states (in this case diamond) is shown in Fig. 3c.

Figure 4a shows horizontal line scans from the π^* and diamond images of Fig. 3a and c. Chemical shifts of the silicon $L_{2,3}$ edge^{24,25} were used to identify the various silicon compounds present (the Si $L_{2,3}$ edge moves from 100 eV in pure Si to 103 eV in a-SiC²⁴ and 106 eV in a-SiO₂ (ref. 25)). Thus, the presence of a-SiC and a-SiO₂ in addition to a-C can be seen in the interlayer region.

Changes in the chemical bonding at the hetero-interface can be identified in Fig. 3. The a-C/diamond interface is rough, but the transition is very sharp, limited to a layer <1 nm thick showing that the diamond nucleated directly on the a-C. In contrast, on specimens prepared under a 250-V negative bias (to increase the nucleation density^{5,6}), diamond was found to nucleate on SiC. In the present work, there is no connection between the SiC layer and the diamond overgrowth, suggesting that the diamond nucleation occurred on the a-C.

It has been postulated that microcrystalline graphite^{10,11}, with a suitable orientation, could be a nucleation site for diamond. In the present study, the presence of such crystallites larger than 1.5 nm in the interlayer could be ruled out from the shape of the carbon K-edge. Instead, analysis¹⁷ of this edge shows $85 \pm 5\%$ of the carbon sites in the interlayer to be three-fold coordinated and the interlayer plasmon energy of 23 eV is typical of sp^2 a-C with little or no hydrogen, that is, similar to that found in grain boundaries^{26,27} of diamond produced by chemical vapour deposition (CVD).

In Fig. 4b, the simultaneously recorded ADF signal is seen to be in good registration with the diamond scan. The full-width half-maximum of the diamond/a-C interface is 1 nm. This suggests that most of the broadening in the energy-loss image is due to specimen tilt as the same broadening is seen in the ADF

image. An upper limit for the spatial resolution of the EELS signal is then the 0.5 nm step size with which both images were recorded. This is consistent with the calculated quantum-mechanical spatial resolution for a 284 eV energy loss of 0.4 nm (ref. 28). The resolution of the ADF signal is 0.3 nm, so differences would arise with a smaller step size.

Because the core edge signal is weaker (for example, by 1,000 times) than the elastic scattering, dwell times of 0.5 to 5 s per pixel can be required to obtain fine-structure detail. Spectra recorded at selected points across an interface have shown changes in the fine structure at the subnanometre scale²⁹. Very recently, Batson³⁰ has investigated changes in the silicon oxidation state across a Si/SiO₂ interface with atomic resolution using a combination of annular dark-field STEM and EELS. Also very recently, Browning *et al.*³¹ have shown that chemical information at a CoSi₂/Si interface can be obtained with atomic resolution perpendicular to the interface by exploiting atomic-column resolution ADF STEM while scanning the beam parallel to the interface to acquire the EELS data. In this way, effective dwell times of 5 s can be achieved for the EELS experiments. However, for a small map (that is, a two-dimensional image) of 64² points, a dwell time of 1 s per pixel is too long to obtain subnanometre resolution due to instabilities in the probe's position, energy and current. Elemental identification is still possible by using a wider energy window and a shorter dwell time thereby giving up the fine structure^{32,33}. Plasmon losses have long been used³⁴ but are intrinsically a low spatial resolution approach²⁸. Further, they primarily measure the valence electron density with no chemical specificity. In the present case, a-C, a-SiC and SiO₂ all have the same plasmon energy and could not be distinguished.

To achieve maps in the core edge fine structure, as demonstrated here, it is necessary to substantially improve both the stability and the detection efficiency of the instrument. This work demonstrates that this is feasible and indeed is the first time that such

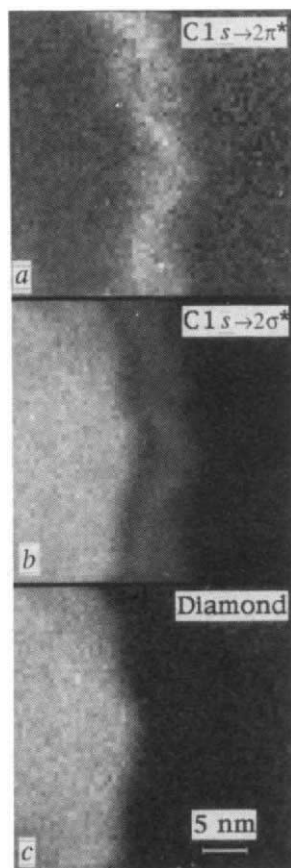


FIG. 3 Energy-selected images of the π^* (a) and σ^* (b) excitations at 285 eV and 290 eV after background subtraction. c, A map of the diamond region, obtained by removing the sp^2 contribution to the σ^* states. This was done by subtracting 1.6 times the $1s \rightarrow 2\pi^*$ image from the σ^* image. The weighting of 1.6 was determined from the areas in the π^* and σ^* windows (see Fig. 1) for sp^2 a-C. A weighting of less than the ideal 2:1 value is to be expected because the band resulting from the σ states is broader than the energy window whereas the π^* band is not. Each pixel is 0.5×0.5 nm and the dwell time was 70 ms per pixel.

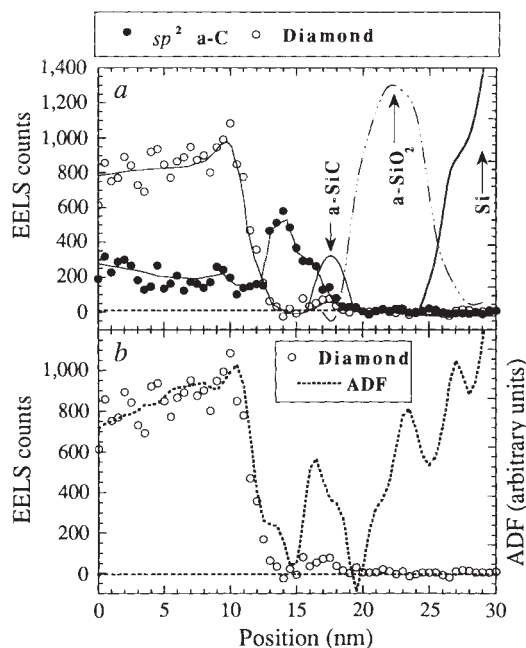


FIG. 4 a, Line scan through Fig. 3 showing the relative intensities of the diamond and sp^2 a-C signals. Also shown are line scans of silicon which were recorded at intervals of 1 nm. The images of carbon were taken with a 0.5×0.5 nm pixel size. The thin layer of a-C on the diamond side is probably surface damage from the ion milling. b, The ADF signal recorded simultaneously with the EELS data. It does not appear to be any sharper than the diamond signal, suggesting that most of the broadening is due to specimen tilt.

two-dimensional and subnanometre resolution images of different bonding states at a buried interface have been presented (as opposed to surface measurements by micro-Raman, Auger electron spectroscopy or X-ray photoelectron spectroscopy which also lack spatial resolution). The LDOS obtained from the EELS near-edge structure can be directly related to electronic structure calculations (at least at a 1-eV resolution) and unlike scanning tunnelling microscopy where only valence states are probed, the mapping of core electron excitations allows elemental identification. □

Received 30 July; accepted 9 November 1993.

1. Davis, R. F. *et al. Mater. Sci. Engng* **B1**, 77–104 (1988).
2. Matsumoto, S., Sato, Y., Kamo, M. & Setaka, N. *Jap. J. appl. Phys.* **21**, L183–L185 (1982).
3. Angus, J. C. & Hayman, C. C. *Science* **241**, 913–921 (1988).
4. Jiang, X., Klages, C. P., Zachai, R., Hartweg, M. & Füsser, H. *J. Appl. Phys. Lett.* **62**, 3438–3440 (1993).
5. Stoner, B. R., Ma, G.-H. M., Woitler, S. D. & Glass, J. T. *Phys. Rev.* **B45**, 11067–11084 (1992).
6. Woitler, S. D., *Appl. Phys. Lett.* **62**, 1215–1217 (1993).
7. Williams, B. E. & Glass, J. T. *J. Mater. Res.* **4**, 373–384 (1988).
8. Pehrsson, P. E., Glesener, J. & Morrish, A. *Thin Solid Films* **212**, 81–90 (1992).
9. Tzou, Y., Bruley, J., Ernst, F., Ruhle, M. & Raj, R. *J. Mater. Res.* (submitted).
10. Lambrecht, W. R. L. *et al. Nature* **364**, 607–610 (1993).
11. Li, Z. *et al. J. appl. Phys.* **73**, 711–715 (1993).
12. Crewe, A. V., Wall, J. & Langmore, J. *Science* **168**, 1338–1340 (1970).
13. Tomboulis, D. H. & Bedo, D. E. *Phys. Rev.* **104**, 590–597 (1956).

14. Colliex, C. & Jouffrey, B. *Phil. Mag.* **25**, 491–511 (1972).
15. Egerton, R. F. *EELS in the Electron Microscope* (Plenum, New York, 1986).
16. Egerton, R. F. & Whelan, M. J. *J. Electron Spectrosc.* **3**, 232–236 (1974).
17. Berger, S. D., McKenzie, D. R. & Martin, P. J. *Phil. Mag. Lett.* **6**, 285–290 (1988).
18. Weng, X., Rez, P. & Sankey, O. F. *Phys. Rev.* **B40**, 5694–5704 (1989).
19. Leapman, R. D., Fejes, P. L. & Silcox, J. *Phys. Rev.* **B28**, 2361–2373 (1983).
20. Vvedensky, D. D. In *Unoccupied Electronic States* (eds Fuggle, J. C. & Inglesfield, J. E. 152 (Topics in appl. Phys. No. 69, Springer, New York, 1992).
21. Silcox, J., Xu, P. & Loane R. F. *Ultramicroscopy* **47**, 173–186 (1992).
22. Kirkland, E. J. *Ultramicroscopy* **32**, 349–364 (1990).
23. Pennycook, S. J. & Boatner, L. A. *Nature* **336**, 565–567 (1988).
24. McKenzie, D. R., Bruley, J. & Smith, G. B. *Appl. Phys. Lett.* **53**, 2284–2286 (1988).
25. Batson, P. E. in *Inst. Phys. Conf. Ser. No. 117*, Section 2 (eds Cullis, A. J. & Long, N. J.) 55–62 (Inst. Phys., Bristol, 1991).
26. Bruley, J., Cuomo, J. J., Guarnieri, R. C. & Whitehair, S. J. *Mater. Res. Soc. Extended Abstr.* **EA-19**, 99–100 (1989).
27. Fallon, P. J. & Brown, L. M. *Diamond and Related Materials* **2**, 1004–1011 (1994).
28. Kohl, H. & Rose, H. *Adv. Electron. electron. Phys.* **65**, 173–224 (1985).
29. Batson, P. E. *Phys. Rev.* **B44**, 5556–5561 (1991).
30. Batson, P. E. *Nature* **366**, 727–728 (1993).
31. Browning, N. D., Chisholm, M. F. & Pennycook, S. J. *Nature* **366**, 143–146 (1993).
32. Mory, C. & Colliex, C. *Ultramicroscopy* **28**, 339–346 (1989).
33. Leapman, R. D. & Hunt, J. A. in *Microscopy: The Key Research Tool* (eds Lyman, C. E., Peachey, L. D. & Fisher, M. J. 39–49 (Electron Microscopy Soc. Am., Woods Hole, 1992).
34. Colliex, C., Krivanek, O. L. & Trebbia, P. in *Inst. Phys. Conf. Ser. No. 61*, Ch 4 (ed. Goringe, M. J.) 183–188 (Inst. Phys., Bristol, 1981).

ACKNOWLEDGEMENTS. Special thanks are due to E. J. Kirkland for his implementation of the STEM data acquisition system and to M. Thomas for maintaining the STEM. This research was supported by the US Department of Energy (D.A.M. and J.S.) and by the US Office of Naval Research (Y.T. and R.R.). Funding for the purchase of the UHV STEM and operation through the Materials Science Center was provided by the US National Science Foundation.

Simultaneous STEM imaging and electron energy-loss spectroscopy with atomic-column sensitivity

P. E. Batson

IBM T. J. Watson Research Center, Yorktown Heights,
New York 10598, USA

IMAGING with the scanning transmission electron microscope (STEM) has recently gained in resolution from the use of the annular dark-field (ADF) method^{1,2}, which produces a signal that is highly sensitive to atomic number. There is a possibility that electrons from the probe beam that are scattered inelastically might be collected to provide a spectroscopic signal containing information about atomic bonding and electronic structure—this is the basis of the standard electron energy-loss spectroscopy (EELS) technique. Browning *et al.*^{3,4} have shown recently that an EELS signal from absorption of atomic core levels can be used, in conjunction with ADF imaging, to obtain information about chemical composition at atomic resolution. Here I report the use of a similar approach to resolve the different bonding states of silicon atoms (Si^0 , Si^{2+} , Si^{4+}) across a Si–SiO₂ interface. The oxidation state of individual columns of unit cells, containing pairs of silicon atoms, can be resolved with this method, allowing the structure of the interface to be characterized in great detail.

In the ADF imaging technique, a sub-0.25-nm diameter probe of 100–300 keV electrons is scanned across an area of single-crystal material that is typically 5–50 nm thick. Large angle elastic scattering is detected and incoherently summed to obtain an imaging signal which depends strongly on the atomic number of atoms illuminated by the probe. Because the nominal probe dimensions are smaller than many interatomic distances, the scattered signal reveals atomic columns with good contrast. The present experiments were intended to assess the strength of the scattering in the soft X-ray region to see if the near-edge fine structure is strong enough to obtain information on bonding and electronic structure. This work used the VG Microscopes

HB501 STEM, modified to include a high-resolution electron spectrometer (70 meV with a ± 20 meV stability and accuracy^{5,6}), a CCD (charge-coupled device)-based electron detector to allow single-electron sensitivity in the EELS, and the VG ultra-high-resolution lens, installed so as to reduce slightly the expected spherical aberration. In addition, the high voltage has been increased to 120 kV. With these modifications, I have obtained a 0.2-nm probe with a measured current of 0.15 nA.

Figure 1 (left) shows a slice of a typical dark-field image of a [110] oriented Si–SiO₂ interface created on an insulating substrate by steam oxidation. The elongated bright structures indicate the position of pairs of Si atoms arranged in columns oriented along the [110] direction perpendicular to the image plane. The image is an average of four 0.5-s exposures. At the Si–SiO₂ interface, I have labelled the columns 1 to 5 for discussion. Column 1 seems from the ADF image to be the first that has a strong excess of Si present, and so probably identifies the interface. The right-hand side of Fig. 1 shows eight spectra for the transition from the 2*p* band to the conduction band (CB) of silicon obtained at the positions indicated in the image. The data were obtained in 7 s total acquisition times with a 50 meV energy step size, using a collection half-angle of 16 mrad. A power-law background intensity has been subtracted, but otherwise the data are as they were measured. These data have been normalized to a standard incident electron dose, so that intensities may be directly compared. In addition to the 0.2-nm probe size, the probe positional accuracy during the measurement was of the order of ± 0.1 nm, so that although these spectra certainly identify scattering due to columns of unit cells (0.53 nm) of Si, they do not yet reflect clean analytical signals from individual columns of atoms.

Within the bulk silicon (layers 3,5), I observe the 2*p*→CB onset for Si^0 at 99.84 ± 0.02 eV with a small contribution from SiO_2 (Si^{4+}) at 106 eV and 108 eV, probably due to a thin native oxide on the top and bottom surfaces of the specimen. The oxide signal does not noticeably increase until layer 2 is reached. Layer 1 has a large contribution from Si^{4+} . In addition, the Si^0 signal is large, possibly due to symmetry-breaking of conduction band states at the interface, allowing transitions to regions of phase space that are normally forbidden, such as the *p*-like final states at the Brillouin zone centre (Γ_2 and $\Gamma_{1,5}$). The increase cannot be due to overlap of signals from elsewhere in the uniformly

thick specimen, because the measured intensity implies a larger Si differential scattering cross-section than is present in the bulk elsewhere. There is also present in layer 1 a clearly visible onset near 103 eV which may be due to Si^{2+} —that is, silicon coordinated by two oxygen and two silicon atoms. This signal has been observed using the spatially resolved partial photo-yield technique of Tonner on a very thin oxide layer on silicon⁷. Although it seems likely that this oxidation state should also exist at the bulk interface, this is the first time that evidence for it has been observed. Far into the SiO_2 , an oxygen sub-stoichiometry is suggested by the presence of a smooth, featureless increase in scattering intensity between the Si onset position at 99.84 eV and the SiO_2 exciton at 106 eV. Occasional Si clustering also seems to be present, suggested by the presence of an identifiable shoulder of intensity at 99.84 eV in the spectra taken 1.0 nm from the interface. This spectral behaviour has been observed in non-stoichiometric oxide films that have been annealed to produce Si clustering⁸. As well as this bonding information, the interface spectra show additional intensity extending below the Si onset at 99.84 eV, possibly due to defect states.

These results show convincingly that EELS spectra are obtainable with good statistics using this probe size, that they certainly change on a length scale commensurate with atomic column spacings, and that bonding and probably electronic structure information is readily obtainable. Some thought needs to be applied to the obtained lateral spatial resolution, however. There is strong evidence⁹ suggesting that the best imaging conditions—those which produce sharp atomic-column images—may not be the best conditions for an EELS measurement. This is due to the presence of undesirable wings of intensity in the probe. These introduce an average background in the image, but will mix the

analytical signal from adjacent columns in the EELS case. It is possible, for instance in these results, that the increased oxide signal in layer 2 is due to a small overlap of the probe intensity with oxide structure in layer 1. It can be said from the present results, however, that the magnitude of the obtainable signal, and the signal contrast—its variation with bonding and electronic structure changes—are both sufficient to reveal rich structure as a function of probe position at the atomic column level.

Very recently, Muller *et al.*¹⁰ have reported the mapping of sp^2 and sp^3 carbon with sub-nanometre resolution at the diamond-silicon interface using a similar combination of STEM and EELS. □

Received 7 September, accepted 9 November 1993.

1. Pennycook, S. J. & Boatner, L. A. *Nature*, **336**, 565–567 (1988).
2. Loane, R. F., Kirkland, E. J. & Silcox, J. *Acta crystallogr.* **A44**, 912–927 (1988).
3. Browning, N. D., Chisholm, M. F. & Pennycook, S. J. *Nature* **336**, 143–146 (1993).
4. Browning, N. D., McGibbon, M. M., Chisholm, M. F. & Pennycook, S. J. in *Proc. 51st Ann. Meeting Microsc. Soc. Am.* (eds Bailey, G. W. & Rieder, C. L.) 576–577 (San Francisco Press, California, 1993).
5. Batson, P. E. *Rev. Sci. Instrum.* **57**, 43–48 (1986).
6. Batson, P. E. *Rev. Sci. Instrum.* **59**, 1132–1138 (1986).
7. Harp, G. R., Han, Z. L. & Tonner, B. P. *Physica Scripta*, **T31**, 23–27 (1990).
8. Dori, L. *et al.* *J. appl. Phys.* **69**, 2317–2323 (1991).
9. Hillard, S., Loane, R. F. & Silcox, J. *Ultramicroscopy* **49**, 14–25 (1993).
10. Muller, D. A., Tzuo, Y., Raj, R. & Silcox, J. *Nature* **366**, 725–727 (1993).

Formation of C_{60} by pyrolysis of naphthalene

Roger Taylor*, G. John Langley†, Harold W. Kroto* & David R. M. Walton*

* School of Chemistry and Molecular Sciences, University of Sussex, Brighton BN1 9QJ, UK

† Chemistry Department, The University, Southampton SO9 5NH, UK

THE formation of bulk quantities of C_{60} by arc discharge between carbon electrodes in an atmosphere of helium¹ or argon^{2,3} has led to an explosion in fullerene research. Methods for improving the rate of fullerene production have included increasing the reactor size and the diameter of the carbon rods, and varying the rate of rod consumption and helium pressure⁴. Systems using several rods have also been employed^{5,6}. The ideal method, however, would involve a continuous process that does not require rod replacement. Approaches using carbon granules⁶ and powders⁷ have been reported, as well as combustion methods using hydrocarbons^{8,9}. Here we report the formation of C_{60} and C_{70} by pyrolysis of naphthalene at $\sim 1,000^\circ\text{C}$. C_{60} and C_{70} are formed by the 'patching together' of six and seven naphthalene molecules respectively, as demonstrated by mass-spectrometric analysis of intermediate products. These results point to a continuous method of fullerene formation, and also show that closed fullerene cages can be built from well defined aromatic fragments.

After the discovery of the spontaneous creation of C_{60} from a chaotic carbon plasma¹⁰, attempts were made to rationalize this process. Zhang *et al.* suggested that polycyclic aromatic networks might form and be driven towards pentagon formation and curling, in order to minimize dangling bonds¹¹. The observation of spheroidal graphite microparticles on pyrolysis of ethylbenzene lead to the suggestion that C_4 chains might be important intermediates¹². More recently, various workers have pinpointed carbon chain/ring species as intermediates in fullerene formation^{13,14}. Goeres and Sedlmayr noted that the 10-carbon naphthalene frameworks could, in principle, be assembled to give C_{60} and C_{70} ^{15,16}. Others have suggested that polycyclic aromatics may be precursors to fullerenes^{17–24}. This suggestion is supported by the observation that if fullerenes are generated from graphite in the presence of hydrogen donors, polycyclic aromatic hydrocarbons are formed^{23,24}. Furthermore, if the arc

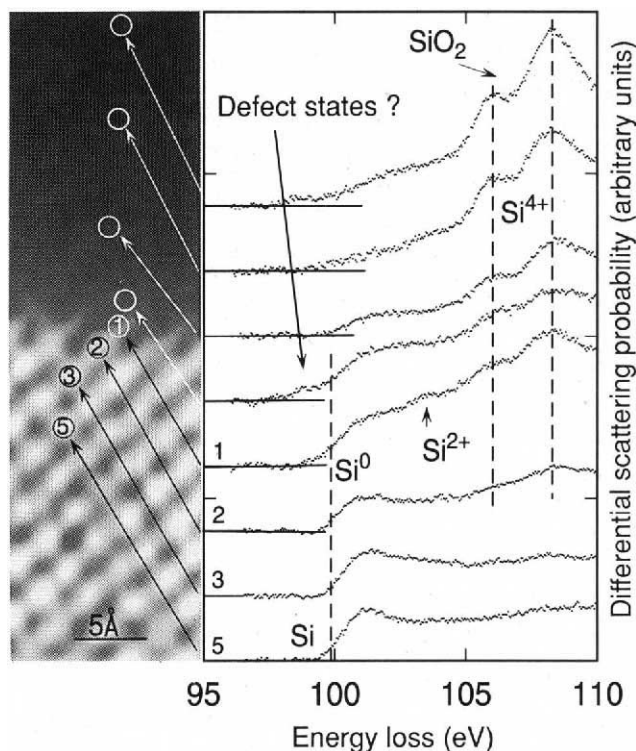


FIG. 1 Left, Incoherent dark-field image of the Si-SiO₂ interface for a steam-formed oxide. The elongated bright structures coincide with chains of Si atom pairs, oriented along the <110> directions. Right, EELS spectra obtained at eight locations indicated by the circles at the left. The bulk Si onset (Si^0) is near 100 eV. The SiO_2 (Si^{4+}) structure lies between 105 and 108 eV. At the interface, a fairly strong Si^{2+} signal is seen for the first time in the bulk. Some structure corresponding to electronic defect states in the silicon gap also appears to be present.

between graphite electrodes is discharged under toluene, both polycyclic aromatic hydrocarbons and C_{60} are produced²⁵. It seemed feasible therefore, that if enough energy could be supplied to break the C-H bonds in naphthalene, assembly of naphthalenoctyl into C_{60} , as in the Schlegel diagram shown in refs 15 and 16, could ultimately result. As it is improbable that all C-H bonds in one naphthalene molecule would break simultaneously, a more reasonable conjecture is that stepwise assembly of partly dehydrogenated units occurs to give intermediate polycyclics such as 11,12-benzofluoranthene, which has a mass-spectrometric signature at a mass/charge ratio of $m/z=252$ (Fig. 1a). The early literature on naphthalene pyrolysis indeed describes formation of hydrogen^{26,27}; especially encouraging was the report, in 1957, of formation of 11,12-benzofluoranthene together with 10,11-benzofluoranthene (Fig. 1b) and perylene (Fig. 1c)²⁸.

In initial experiments, naphthalene was deposited at the inlet end of a silica tube 1 cm in diameter and 40 cm long. The outlet led into a dry-ice-cooled glass coil and then a series of acetone bubblers. Argon (at atmospheric pressure) was passed through the apparatus at 15 ml min^{-1} for a few minutes, and then a 3-cm length of the silica tube near the outlet end was heated with a butane torch. The naphthalene deposit was then gently heated with a second torch and pyrolysis occurred as the vapour passed through the heated zone. The heated tube glowed much more strongly each time the naphthalene stock was heated, suggesting that an exothermic reaction was occurring. The contents of the tube and cooling coil were removed with acetone, and combined with the acetone solution from the bubblers. The product was yellow-orange, and the absence of acetone-insoluble material indicated that fullerenes had not formed. This was confirmed by the mass spectrum obtained under electron-impact conditions at 70 eV, 6 kV accelerating voltage, 350°C source temperature, and a direct chemical ionization probe ramped to 1 mA ($\sim 650^\circ\text{C}$). A blank spectrum was obtained immediately before the run, to ensure the absence of fullerene contamination. Figure 2a-c, obtained with increasing probe temperature, reveals the following significant features: (1) $m/z=254$, 252. Binaphthyl, followed by condensation to give a mixture of benzofluoranthenes (Fig. 1a, b) and perylene (Fig. 1c), reported previously²⁶. Two to four hydrogens eliminated. (2) $m/z=380$, 378, 376. Trinaphthyl, and then further hydrogen elimination giving first naphthyl joined to either benzofluoranthene or perylene, and then further ring fusion. Four to eight hydrogens eliminated. (3) $m/z=506$, 504, 502, 500, 498. Tetranaphthyls, and then further condensation giving first binaphthyl joined to either benzofluoranthene or perylene, followed by further couplings and ring closures to give two-thirds of the C_{60} framework for example, Fig. 3. 6-14 hydrogens eliminated. (4) $m/z=632$, 630, 628. Pentanaphthyls, and then further condensation to give structures some of which must be non-planar. 8-12 hydrogens eliminated. (5) $m/z=758$, 756, 754. For example, tetranaphthylbenzofluoranthene, and further ring closure. 10-14 hydrogens eliminated. (6) $m/z=882$. Various combinations of naphthalene moieties, but one pair must be fully fused, as in benzofluoranthene or perylene.

The experiment was then repeated, the output end of the silica tube being heated (with a propane-oxygen torch) to $\sim 1,000^\circ\text{C}$

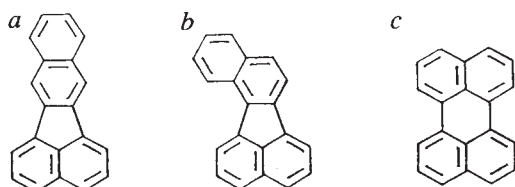


FIG. 1 a, 11,12-Benzofluoranthene. b, 10,11-Benzofluoranthene. c, Perylene.

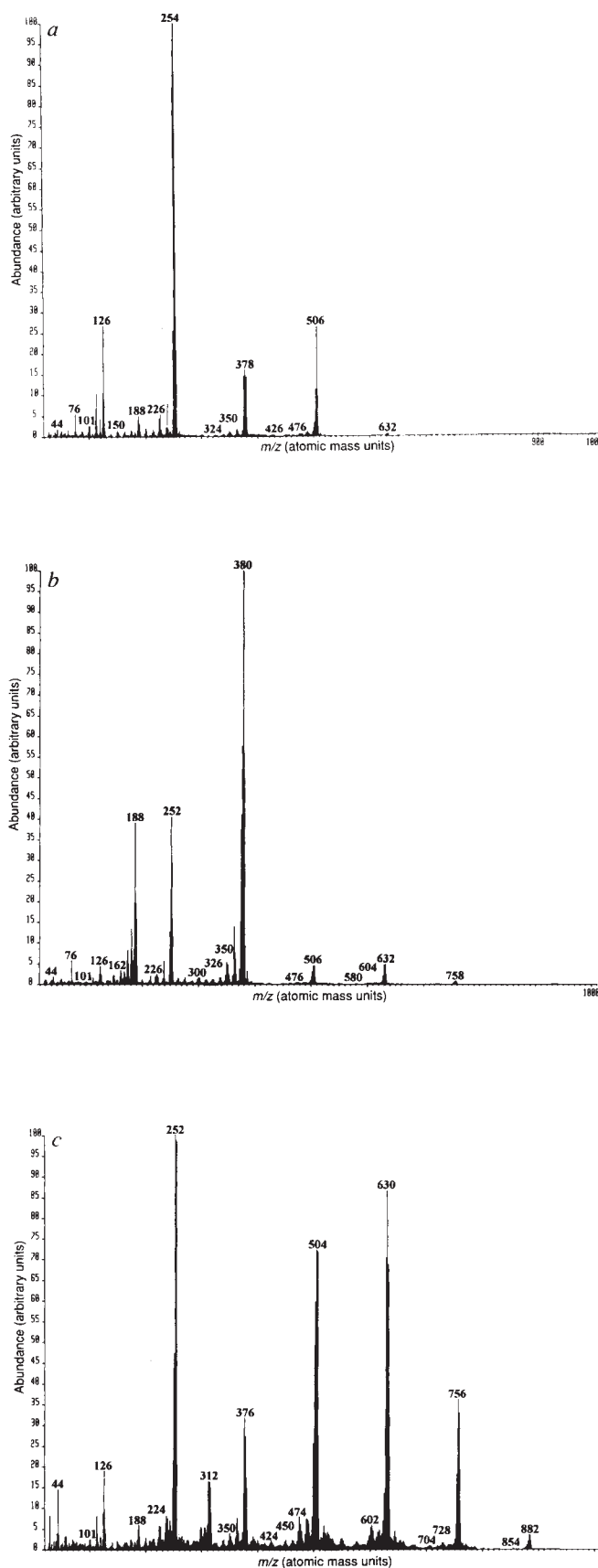


FIG. 2 a-c, Mass spectra of the products of pyrolysis of naphthalene at $\sim 500^\circ\text{C}$. a-c, Are the spectra obtained with increasing temperature of the direct chemical ionization probe (see text). (m/z is the mass/charge ratio.) (See Supplementary Information.)

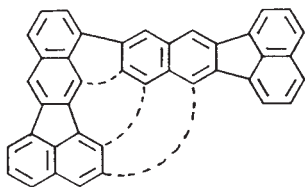


FIG. 3 Proposed bis-benzofluoranthyl structure (solid lines) for the species giving $m/z=502$; dotted lines indicate how further eliminations give the species of $m/z=496$.

(as measured by a Pt/PtRh thermocouple). Extensive decomposition occurred, and sooty material was deposited both in the cold trap and the acetone bubblers. The acetone slurry of the products was filtered and washed thoroughly with acetone, leaving a black deposit which, when washed with carbon disulphide gave a deep red solution, and a black graphitic residue; a carbon deposit, metallic in appearance, formed on the heated surface of the silica tube.

The mass spectrum (Fig. 4a, b) of the black solid obtained from the red CS_2 solution by evaporation showed the following main features: (1) $m/z=376$, 374. Further elimination from the

naphthylbenzofluoranthenes or naphthoperylene isomers inferred from the mass spectrum obtained at lower temperature. 8 and 10 hydrogens eliminated. (2) $m/z=500$, 498, 496. Ring fusion, additional to that observed at lower temperature. Up to 16 hydrogens eliminated. (3) $m/z=624$, 622, 620. Further fusion of, for example, benzofluoranthenes with naphthalene, followed by two further ring closures with up to 20 hydrogens eliminated. These species (which must be non-planar) now show C_2 fragmentation loss, characteristic of fullerenes. (4) $m/z=752$, 750, 748, 746. Various ring closures of the structure which gave $m/z=754$ at lower temperature, up to 22 hydrogens being eliminated. A typical set of ring closures required to make C_{60} from three fused 11,12-benzofluoranthene molecules is shown in Fig. 5; even 'linear' naphthobenzofluoranthenes, which cannot undergo intramolecular dehydrogenation, can combine like two halves of a tennis ball to form C_{60} . (5) $m/z=\sim 738$. There is a cluster of ions at this mass charge ratio (some having undergone further elimination), suggesting that this is the most stable, least hydrogenated precursor of C_{60} . Significantly, these species are not present in the product that did not contain C_{60} . (6) $m/z=720$. C_{60} , showing the characteristic lack of $m-24$ (that is, C_2) fragments; the extract also showed the infrared bands at 527 and 577 cm^{-1} seen previously for C_{60} (ref. 29). (7) $m/z=840$. C_{70} , the mode of formation of which may involve perylene (Fig. 1c), or simple isomerization of the 70-carbon species as proposed

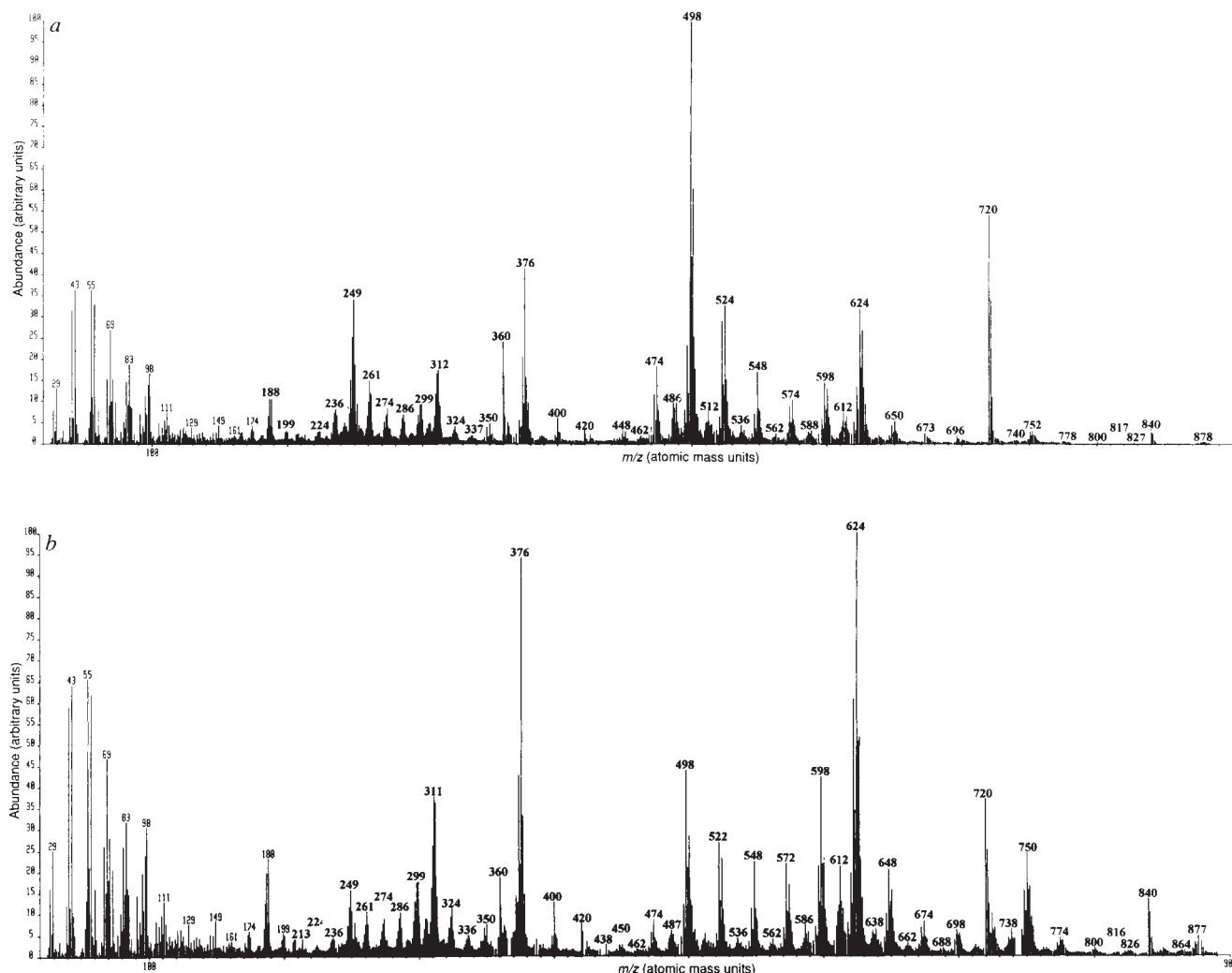


FIG. 4 a, b, Mass spectra of the products of pyrolysis of naphthalene at $\sim 1,000^\circ\text{C}$. a, b, Are the spectra obtained with increasing temperature of the direct chemical ionization probe (see text). (See Supplementary Information.)

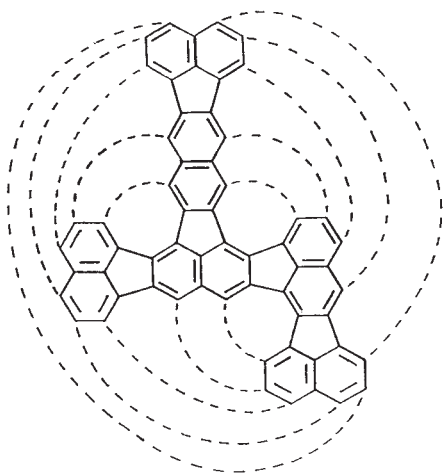


FIG. 5 An example of formation of C_{60} by ring closures from three fused 11,12-benzofluoranthene molecules.

in ref. 15. Other results (not shown) indicate that hydrogenated species of $m/z \sim 850$ may be the most stable immediate precursors of C_{70} .

The fullerene yields are so far $<0.5\%$ (based on naphthalene consumed) and are variable; the highest-yield runs give the deepest-red solution (in CS_2). If the rate of naphthalene feed into the heated zone is too rapid then no fullerene is formed. This suggests that under optimum conditions, yields may be raised to a viable level. Moreover the acetone-soluble byproducts, which contain the fullerene precursors, should be recyclable to increase the yield.

The results show that in our process at least, C_{60} is assembled through successive combination of C_{10} fragments. These may proceed to a hydrogenated 60-carbon species which then eliminates hydrogen. For example, in the $C_{60}H_{28}$ species (Fig. 5), as each 'inner' pair of hydrogens is eliminated, the molecule curls, bringing closer together the next pair of hydrogens to be eliminated. Intramolecular hydrogen elimination from the intermediates may also occur at any stage, leading ultimately to a partially hydrogenated 60-carbon species, followed by hydrogen elimination. The low temperature of the pyrolysis method compared to that in the arc-process ($\sim 3,500^\circ C$) suggests that assembly of C_2 species into C_{10} sub-units is the process of highest activation energy. We note that C_{10} species exhibit the highest intensities amongst neutral C_n clusters in Rydberg-electron mass spectrometry (though this result may partly reflect a large electron-capture cross section)³⁰.

We believe that the observation of a cluster of ions around $m/z = 738$ is relevant to the detection of a species corresponding to $C_{60}H_{18}$ (along with $C_{60}H_{36}$) in the mass spectra of the products of C_{60} reduction under Birch conditions (Na/NH_3) and by 9,10-dihydroanthracene^{31, 34}. Although one may conceive of symmetrical structures corresponding to $C_{60}H_{36}$ (see, for example, ref. 34), this is not the case for $C_{60}H_{18}$. The possibility exists therefore that $C_{60}H_{18}$ present in the products of these reductions is a cage-opened species.

By use of appropriate aromatic hydrocarbons, it may prove possible to 'tune' the process to produce a particular fullerene. It may also be possible to generate endohedral fullerenes by pyrolysis over appropriate oxides. □

Received 25 August; accepted 22 November 1993.

- Krätschmer, W., Lamb, L. D., Fostiropoulos, K. & Huffman, D. R. *Nature* **347**, 354–358 (1990).
- Taylor, R., Hare, J. P., Abdul-Sada, A. K. & Kroto, H. W. *J. chem. Soc., chem. Commun.* 1423–1425 (1990).
- Hare, J. P., Kroto, H. W. & Taylor, R. *Chem. Phys. Lett.* **177**, 394–398 (1991).
- Parker, D. H. et al. *Carbon* **30**, 1167–1182 (1992).

- Olah, G. A. et al. *J. Am. chem. Soc.* **113**, 9385–9387 (1991).
- Withers, J. C. presented at *Electrochemical Soc. Meeting*, Hawaii (1993).
- Yoshie, K., Kasuya, S., Eguchi, K. & Yoshida, T. *Appl. Phys. Lett.* **61**, 2782–2783 (1992).
- Howard, J. B., McKinnon, J. T., Makarovskiy, Y., LaFleur, A. L. & Johnson, M. E. *Nature* **352**, 139–142 (1991); *J. phys. Chem.* **96**, 6657–6662 (1992).
- McKinnon, J. T., Bell, W. L. & Barkly, R. M. *Combust. Flame* **88**, 102–103 (1992).
- Kroto, H. W., Heath, J. R., O'Brien, S. C., Curl, R. F. & Smalley, R. E. *Nature* **318**, 162–163 (1985).
- Zhang, Q. L. et al. *J. phys. Chem.* **90**, 525–528 (1986).
- Kroto, H. W. & Walton, D. R. M. in *Carbocyclic Cage Compounds* (eds Osawa, E. & Yonemitsu, O.) 91–100 (VCH, New York, 1992).
- McElvaney, S. W., Ross, M. A., Goroff, N. S. & Diederich, F. *Science* **259**, 1594–1596 (1993).
- von Helden, G., Hsu, M.-T., Kemper, P. R. & Bowers, M. T. *J. phys. Chem.* **95**, 3835–3837 (1991).
- Goeres, A. & Sedlmayr, E. *Chem. Phys. Lett.* 310–317 (1991).
- Broyer, M. et al. *Chem. Phys. Lett.* **198**, 128–134 (1992).
- Herndon, W. C. & McKee, M. L. *J. molec. Struct.* **153**, 75–80 (1987).
- Rubin, Y., Kahr, M., Knobler, C. B., Diederich, F. & Wilkins, C. L. *J. Am. chem. Soc.* **113**, 495–500 (1991).
- Eletsii, A. V. & Smirnov, B. M. *Soviet Phys. Usp. (Engl. Transl.)* **34**, 616–625 (1991).
- Diederich, F. & Rubin, J. *Angew. Chem. Int. Edn. engl.* **31**, 1101–1123 (1992).
- Taylor, R. *J. chem. Soc., Perkin Trans. 2*, 1667–1669 (1992).
- Plater, M. J. *Synlett*, 405–406 (1993).
- Chang, T. M., Naim, A., Ahmed, S. N., Goodloe, G. & Shevlin, P. B. *J. Am. chem. Soc.* **114**, 7603–7604 (1992).
- Greenwood, P. F. & Hatcher, P. G. *Org. Mass Spectrom.* **28**, 291–292 (1993).
- Beck, M. T., Dinya, Z., Keki, S. & Papp, L. *Tetrahedron* **49**, 285–290 (1993).
- Tilcheev, M. D. & Shchitkov, V. K. *Chem. Zentrbl.* **1**, 4583–4587 (1937).
- Kinney, C. R. & del Bel, E. *Ind. Engng Chem.* **46**, 548–550 (1954).
- Lang, K. F., Buffleb, H. & Kalow, J. *Chem. Ber.* **90**, 2888–2893 (1957).
- Hare, J. P. et al. *J. chem. Soc., chem. Commun.* 412–413 (1991).
- Carman, H. S. & Compton, R. N. *J. chem. Phys.* **98**, 2473–2476 (1993).
- Haefliger, R. E. et al. *J. phys. Chem.* **94**, 8634–8635 (1990).
- Banks, M. R. et al. *J. chem. Soc., chem. Commun.* 1149–1152 (1993).
- Rüchardt, C. et al. *Angew. Chem. Int. Edn. engl.* **32**, 584–586 (1993).
- Austin, S. J., Batten, R. C., Fowler, P. W., Redmond, D. B. & Taylor, R. *J. chem. Soc., Perkin Trans. 2*, 1383–1386 (1993).

SUPPLEMENTARY INFORMATION. Full-size copies of these mass spectra are available. Requests should be addressed to Mary Sheehan at the London editorial office of *Nature*.

U–Pb ages of single shocked zircons linking distal K/T ejecta to the Chicxulub crater

Thomas E. Krogh*, Sandra L. Kamo*,
Virgil L. Sharpton†, Luis E. Marin‡
& Alan R. Hildebrand§

* Jack Satterly Geochronology Laboratory, Royal Ontario Museum, 100 Queen's Park, Toronto, Ontario M5S 2C6, Canada

† Lunar and Planetary Institute, 3600 Bay Area Boulevard, Houston, Texas 77058, USA

‡ Instituto de Geofísica, UNAM, Mexico City, CP 04510, Mexico

§ Geological Survey of Canada, 1 Observatory Crescent, Ottawa, Ontario K1A 0Y3, Canada

MANY lines of evidence now support the identification of the Chicxulub structure, in Mexico, as a buried impact crater and the probable source of ejecta found in Cretaceous/Tertiary (K/T) boundary sections worldwide^{1–8}. The question remains, however, of whether there might be additional craters of K/T age, which also contributed ejecta. Shocked zircons from a K/T ejecta layer in Colorado⁹ preserve a primary source age of 545 ± 5 Myr, with variable degrees of isotopic resetting consistent with partial lead loss at the time of impact (65 Myr ago)¹⁰. Here we report that the impact breccia from Chicxulub contains zircons that are identical in age, texture and lead loss pattern to the Colorado zircons, and to some from the Beloc section in Haiti. U–Pb data for 18 of the 36 grains studied (from all three sites) fall on a single line as well as if they had come from a single sample. An additional source age of 418 ± 6 Myr is found only in Chicxulub and Haiti, suggesting that the Haiti and Colorado ejecta might have sampled different parts of the target stratigraphy. As all the ejecta ages found so far are also found at Chicxulub, our results are consistent with this being the only significant continental K/T impact crater.

Our study was carried out on zircons from the Beloc site (no. 1) in Haiti¹¹, and on a 30-g carbonate-rich polymict breccia sample (with 20–25% of clasts, consisting of 0.2–2.0 mm silicate basement rocks) from the Y6 drill hole (sample N13, from a depth of 1,100–1,103 m), located within and 50 km southwest of the centre of the Chicxulub structure^{5,7,12}; the results are compared to those for zircons from K/T boundary distal ejecta from the Raton basin in Colorado¹⁰. The breccia contains a variety of lithic fragments⁷, so our sample may contain a mixture of zircons typical of the entire crater of excavation. Zircons in Colorado and Haitian ejecta should consist of a similar mixture. Grains from all sites should record the primary age(s) of the target basement, as well as the physical and isotopic changes induced during the short (10^2 s) interval between impact initiation and dispersal above the upper atmosphere. Age variations are expected, given the large size of the transient cavity (diameter estimated at both 90 km and 170 km^{5,13}) and the fact that even a short-lived crust-forming event may span 50–100 Myr.

Basement ages at the Chicxulub site have not been previously determined, but a two-stage evolution is implied by the presence of both foliated granitic gneiss and quartz-mica schist in the Y6 breccia⁷, and only weakly metamorphosed rhyolite porphyry¹² in drill hole Y1, located ~165 km southeast of the crater's centre¹³. The latter site has an estimated age of 410 ± 15 Myr, based on a two-point whole-rock Rb–Sr isochron (R. Zartman, personal communication). Granitic gneiss can be expected to contain euhedral to metamorphically resorbed zircons, whereas any clastic sediment present at the time of impact⁵ would probably contain highly rounded grains; both types are present. The latter should be more susceptible to lead loss due to radiation damage that accumulates at low surface temperatures, whereas grains from deep in the crust may have been spontaneously annealed, and therefore initially concordant and less reset upon impact. All grains for analysis were first selected under a binocular microscope, where grains with planar deformation features (PDF) and granular (or polycrystalline) texture⁹ generally appear translucent to milky. Before analysis, each grain was examined by electron microprobe to confirm its composition and to record shock features.

Determining precise U–Pb zircon ages for single 1–4 μg grains of geologically young material is analytically challenging. Samples are smaller than previously analysed and contain only 5–200 picograms (1 pg is 1×10^{-12} g) of radiogenic lead and therefore low-contamination methods (2 ± 1 pg laboratory lead background) are essential. Owing to the refractory nature of zircon, several days in a pressure vessel (in HF and HNO_3) at 220 °C were required to dissolve the grains¹⁴. The importance of reducing the lead background, in order to maximize the amount of radiogenic lead above background, is demonstrated clearly by the size of the error ellipses in Fig. 1 for analyses CH1 (Table 1), and C3 and C2 from our previous study¹⁰. These samples are 9%, 50% and 91% radiogenic, respectively, for the least abundant (^{207}Pb) lead isotope and, because the exact isotopic composition of background lead is unknown, errors increase in proportion to the size of this correction. The uncertainties ('conventional errors') shown in Fig. 1 and Table 1, and used in the regression analyses unless

otherwise stated, are calculated by combining measurement errors with expected variations in fractionation, and the isotopic composition of laboratory and sample common lead. Smaller errors (in brackets in Table 1) are based on reproducibility estimates, adjusted upward as the per cent radiogenic ^{207}Pb decreases. These smaller (minimum) errors might be valid for samples with similar common lead and fractionation, provided measurement errors are small. We use these minimum errors in some cases as a more rigorous test of collinearity, in an attempt to identify grains with an identical primary age. In the following discussion, we show that various groupings of selected data form linear arrays and we give the probability that the observed scatter is due to the conventional errors alone¹⁵. We do this not to prove a highly precise age, but rather to show how many analyses fall on a single line as well as could be expected for grains from a single rock. For such a demonstration, we normally accept any combined probability in excess of 10%, as this value can be expected one time out of ten; as for individual probabilities, it is not surprising to see some points with only a few per cent probability.

The results for 36 analysed zircons (14, 15 and 7 analyses for Colorado (C), Chicxulub (CH) and Haiti (H), respectively) define a remarkably simple age pattern. A primary (crystallization) age of 544.5 ± 4.7 Myr (Fig. 1 and Table 1) is indicated by 18 analyses (11, 5 and 2 analyses from three sites, respectively) that are collinear with an 89% probability of fit; hence, basement of this age must be the most common zircon-bearing component at the impact site. If only the data for the Colorado site¹⁰ (our largest data set) are considered, 10 analyses give an age of 544.0 ± 5.2 Myr with a 51% probability of fit. The upper part of this line, thickened to include the ± 5 Myr 2σ error and showing

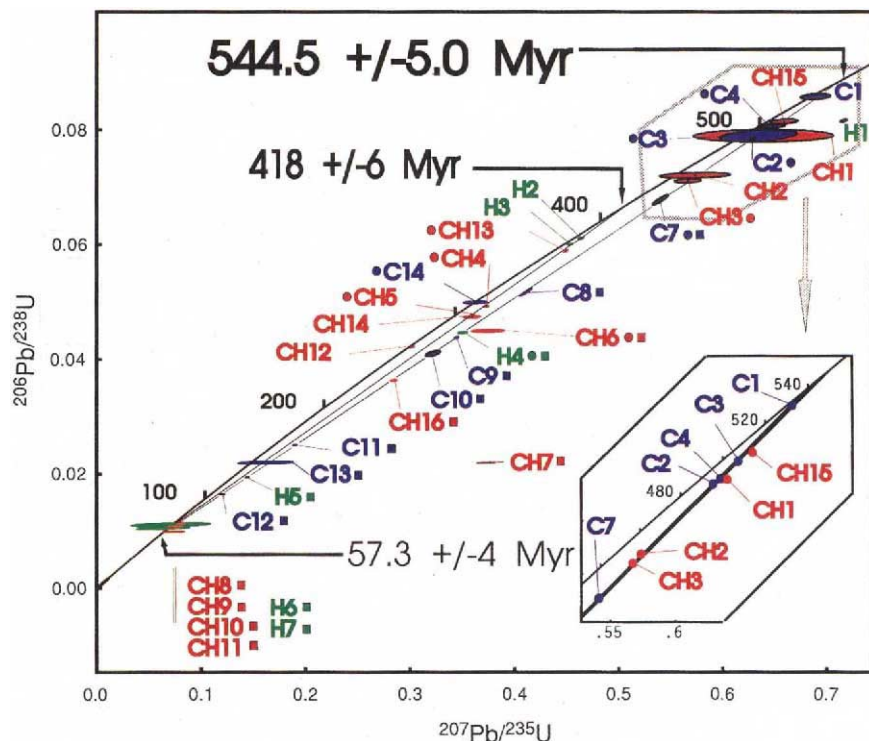


FIG. 1 Concordia diagram for U–Pb data for shocked zircons from an impact breccia from the Chicxulub crater (CH), distal ejecta (fireball layer) from the Raton basin, Colorado (C) and proximal ejecta with possible fireball components from Haiti (H). A dominant source age of 545 ± 5 Myr is indicated for Colorado and Haitian ejecta and the Chicxulub crater, with a minor 418 ± 5 Myr component at Chicxulub and Haiti. Symbol beside sample number indicates the degree of shock-induced textural change that corresponds to discordance: solid squares denote grains with a granular texture, solid circles denote grains with planar deformation features, the least shocked near concordant grains are unmarked. Inset shows near concordant data for grains from Colorado and Haiti, without errors, for comparison. Data for sample C5 and C6 (in ref. 10) are imprecise due to a very low radiogenic content and are omitted for clarity.

TABLE 1 U–Pb data for zircons and a titanite grain

(a) U–Pb data for shocked and unshocked zircons and an unshocked titanite grain from a Chicxulub breccia														
Grain number	Grain description	Weight (mg)	Pb (p.p.m.)	U (p.p.m.)	Th (p.p.m.)	Pb* (pg)	(%) ²⁰⁷ Pb [†]	²⁰⁷ Pb/ ²⁰⁴ Pb [‡]	²⁰⁶ Pb/ ²⁰⁴ Pb [§]	²⁰⁶ Pb/ ²³⁸ U	²⁰⁷ Pb/ ²³⁵ U	²⁰⁷ Pb/ ²⁰⁶ Pb	²⁰⁶ Pb/ ²³⁸ U Age (Myr)	²⁰⁷ Pb/ ²⁰⁶ Pb Age (Myr)
CH1	UNSHOCKED, one face rounded and one angular, coarse fractures, colourless, clear in parts some milky patches, 127 µm	0.001	81	294	420	53	8.8	17.1	45	0.07893 ± 0.69	0.63991 ± 5.25 (±5.00)	0.05880 ± 5.25 (±1.00)	489.7 ± 3.2	560 ± 100
CH2	Unclear if shocked, no faces, subrounded, cracked, translucent, partly milky, 112 µm	0.001	14	125	70	6	25.7	21.0	113	0.07204 ± 0.35	0.57400 ± 2.95 (±1.71)	0.05779 ± 2.86 (±0.34)	448.4 ± 1.5	522 ± 61
CH3	PDF, subrounded, smooth surfaces, opaque, 100 µm	0.002	13	159	157	2	72.5	56.8	734	0.07107 ± 0.24	0.56727 ± 1.11 (±0.061)	0.05789 ± 1.06 (±0.12)	442.6 ± 1.0	526 ± 23
CH4	PDF, euhedral, flat tabular shape, milky, cracked, 110 µm	0.001	104	1,899	1,058	6	77.1	69.3	981	0.04926 ± 0.15	0.37331 ± 0.34 (±0.57)	0.05496 ± 0.30 (±0.12)	310.0 ± 0.5	411 ± 7
CH5	PDF (one direction) and possibly granular, angular fragment, milky, cracked, 160 µm	0.001	147	2,434	2,733	4	86.0	111.1	1,771	0.04775 ± 0.14	0.36022 ± 0.27 (±0.51)	0.05471 ± 0.23 (±0.11)	300.7 ± 0.4	400 ± 5
CH6	Granules appear to be developing, subrounded zircon tip, translucent to milky, 130 µm	0.002	7	139	45	7	0.4	22.4	132	0.04489 ± 0.25	0.37599 ± 2.06 (±1.45)	0.06075 ± 2.01 (±0.30)	283.7 ± 0.7	630 ± 43
CH7	GRANULAR, tabular shape, rounded edges, milky to opaque, 118 µm	0.001	10	351	11	4	49.0	30.6	140	0.02201 ± 0.35	0.37701 ± 1.71 (±0.90)	0.12422 ± 1.58 (±0.18)	140.4 ± 0.5	2,018 ± 28
CH8	GRANULAR, equant, short prisms, well rounded, very smooth surface, opaque 125 µm	0.001	12	960	201	2	56.3	35.7	413	0.01138 ± 0.29	0.08064 ± 2.26 (±0.78)	0.05138 ± 2.19 (±0.15)	73.0 ± 0.2	258 ± 49
CH9	GRANULAR, well rounded, very smooth shiny surface, opaque, cracked, 1 µm granules are rounded, 164 µm	0.003	2	215	27	2	42.2	27.0	246	0.01068 ± 0.25	0.07467 ± 3.86 (±1.04)	0.05070 ± 3.75 (±0.21)	68.5 ± 0.2	227 ± 84
CH10	GRANULAR, euhedral except one rounded face, milky, some hollow granules, 160 µm	0.002	5	443	73	2	48.0	29.8	297	0.01066 ± 0.21	0.07566 ± 2.93 (±0.92)	0.05146 ± 2.84 (±0.19)	68.4 ± 0.1	261 ± 64
CH11	GRANULAR, well rounded grain, broken surface shows granules developed through grain, opaque, 142 µm	0.003	2	137	15	3	24.6	20.7	114	0.00988 ± 0.40	0.07399 ± 6.60 (±1.79)	0.05434 ± 6.40 (±0.36)	63.4 ± 0.3	385 ± 100
CH12	UNSHOCKED TITANITE, large angular fragment, 362 × 180 µm	0.022	9	200	67	17	69.4	51.0	703	0.04223 ± 0.19	0.30231 ± 0.40 (±0.63)	0.05192 ± 0.36 (±0.12)	266.6 ± 0.5	282 ± 8
CH13	PDF, angular fragment, milky, smooth polished surface, coarse cracks, 160 µm	0.003	63	860	907	8	81.1	82.7	1,236	0.05896 ± 0.13	0.44974 ± 0.25 (±0.54)	0.05533 ± 0.21 (±0.11)	369.3 ± 0.5	425 ± 5
CH14	UNSHOCKED, long prisms, subrounded, smooth shiny surface, clear, 130 µm	0.001	22	414	317	2	73.4	58.7	805	0.04744 ± 0.21	0.36017 ± 1.08 (±0.60)	0.05506 ± 1.03 (±0.12)	298.8 ± 0.6	415 ± 23
CH15	UNSHOCKED, subhedral, faces well preserved, colourless, clear, smooth shiny surface, 122 µm	0.001	30	324	215	3	67.3	47.7	568	0.08144 ± 0.25	0.65954 ± 1.03 (±0.65)	0.05874 ± 0.97 (±0.12)	504.7 ± 1.2	557 ± 21
CH16	GRANULAR, euhedral, equant shape, zircon tip, opaque, 100 µm	0.003	18	452	136	3	81.4	83.7	1,200	0.03634 ± 0.17	0.28506 ± 0.54 (±0.54)	0.05689 ± 0.50 (±0.11)	230.1 ± 0.4	487 ± 11
(b) U–Pb data for zircons from a tektite layer in Haiti														
H1	Unclear if shocked, rounded tips, faces preserved, varies from translucent to milky, 2:1 prism 145 µm	0.003	36	551	178	2	94.6	289.1	4,326	0.08160 ± 0.15	0.71702 ± 0.22 (±0.50)	0.06373 ± 0.17 (±0.10)	505.7 ± 0.7	733 ± 4
H2	Unclear if shocked, rounded fragment, coarse fractures, milky to opaque, 160 µm	0.004	44	472	976	5	82.7	89.9	1,373	0.06112 ± 0.14	0.46403 ± 0.29 (±0.50)	0.05507 ± 0.25 (±0.10)	382.4 ± 0.5	415 ± 5
H3	Possible PDF (one direction), rounded tips otherwise euhedral, clear, some cracks, 25:1 prism 135 µm	0.003	39	638	246	2	94.2	268.1	4,640	0.06006 ± 0.15	0.45415 ± 0.23 (±0.50)	0.05484 ± 0.19 (±0.10)	376.0 ± 0.6	406 ± 4
H4	PDF and GRANULAR, euhedral and translucent where PDF, milky, cracked, rounded where granular, 1.5:1 prism 165 µm	0.002	31	579	213	8	60.0	38.7	425	0.04465 ± 0.14	0.35095 ± 0.61 (±0.73)	0.05701 ± 0.58 (±0.15)	281.6 ± 0.4	492 ± 13
H5	GRANULAR, well-rounded flat oval, coarse cracks, opaque, 2:1 prism 180 µm	0.004	20	691	416	5	70.0	52.0	698	0.01944 ± 0.16	0.14420 ± 0.54 (±0.63)	0.05379 ± 0.51 (±0.13)	124.1 ± 0.2	362 ± 11
H6	GRANULAR, rounded, opaque 2:1 prism 105 µm	0.002	2	59	113	4	4.9	16.4	37	0.01115 ± 1.37	0.07143 ± 26.2 (±8.98)	0.04645 ± 25.5 (±1.80)	71.5 ± 1.0	21 ± 100
H7	GRANULAR, very well rounded, opaque, 3:1 prism 145 µm	0.003	1	63	5	4	7.7	16.9	49	0.01045 ± 0.96	0.06448 ± 19.2 (±5.71)	0.04476 ± 18.6 (±1.14)	67.5 ± 0.6	

Zircons vary from clear (unshocked) to translucent or milky (with PDF), to opaque white (granular texture). PDF, planar deformation feature (see text). The longest dimension of the grain is given. Errors on the Pb/U and ²⁰⁷Pb/²⁰⁶Pb ratios are per cent 2σ larger errors (see text); errors in brackets are minimum errors (see text). Due to uncertainties in sample weight, absolute concentrations of U and Pb may be in error by up to 50%. All measurements were made using a Daly detector calibrated against a Faraday cup.

* Total common Pb from sample and laboratory procedures.

† Percent radiogenic ²⁰⁷Pb.

‡ Measured ratio.

§ Corrected for spike and fractionation (0.1% ± 0.038% and 0.1% ± 0.026% per a.m.u. for Pb and U, respectively). Fractionation corrections for large and small samples overlap, indicating that variations in fractionation are insignificant.

|| Corrected for spike, fractionation, blank (2 pg for Pb; 0.5 pg for U), and initial common Pb using a Pb evolution model¹⁶.

the distribution of Chicxulub and Colorado data (without errors for clarity), is shown as an inset of Fig. 1. Statistical tests show that data for five Chicxulub zircons (CH1–3, 15, 16) fall on this line with individual probabilities (using conventional errors) of 12%, 70%, 72%, 12% and 4% respectively, as do the data for two zircons from Haitian ejecta (H4, 5), with 37% and 28% probability. If we assume a lower intercept of 65.5 ± 3 Myr (the best estimate¹⁰ for the time of impact-related lead loss from the Colorado zircons, and consistent with the $^{70}\text{Ar}/^{39}\text{Ar}$ ages of Chicxulub melt rocks^{6,7}), the four most concordant Chicxulub data give an age of 550 ± 15 Myr with a 47% probability of fit. The Colorado data may include components of slightly different age because, using minimum errors (see above), the upper five data points project to 544 ± 4 Myr (81% probability of fit) whereas four of the five lower points (C8–11) project to 559 ± 5 Myr (35% probability of fit). A similar age bimodality is suggested for the Haitian and Chicxulub populations because analyses H4, 5, CH15, 16 combined with lower Colorado points give an age of 556 ± 10 Myr with an 87% probability of fit (for conventional errors). Adding analyses for Chicxulub grains (CH1–3, 15, 16) to the five upper Colorado points (C1–4, 7) gives the younger age of 540 ± 6 Myr with a 99.5% probability of fit. Thus, material at the target site may have two slightly different ages, one at 544 ± 5 Myr, the other at 559 ± 5 Myr. With few exceptions, the most concordant data for each of the three sites give the younger age, whereas the older age is given by the most discordant results. These ages also correspond to weakly to moderately shocked (PDF) grains versus the highly transformed polycrystalline grain types⁹, and hence the rocks closest to the point of maximum shock pressure may have a slightly greater age than those further out. Alternatively, but less probably, the most shocked grains may have undergone a small but systematic amount of recent lead loss. The combination, in a single regression, of near-concordant data with a young apparent primary age, and more-discordant data with an older age, results in a projected lower intercept at 57 ± 4 Myr, which is slightly younger than expected^{6,7,10}. Thus, complexities due to possible mixed-age populations and possible lead loss pose problems for the precise determination of the lower intercept, and hence the impact age. In future, we hope to determine a better U–Pb age for the K/T impact by dating zircons from volcanic ash bounding the ejecta layer or from Chicxulub melt rock, when samples become available.

Data for two grains from Haiti (H2, 3) and three from Chicxulub (CH4, 5, 14) fall on a second line with a primary age of 418 ± 6 Myr and a 36% probability of fit. If analysis CH13 is added, the probability of fit falls to 2%, so that this point is close to but not precisely on this line. This age component is found only in Haitian ejecta and in the Chicxulub breccia, so rock of this age may be restricted to the side of the crater near this site, where it was picked up as ballistic ejecta but was not included in the distal Colorado 'fireball' ejecta.

Remarkably, only 6 of the 36 zircons analysed give ages that are not consistent (using conventional errors and projecting from 65.5 Myr) with the two described above (excluding six totally reset grains). Of these, four fall within error of the 800–500 Myr range typical of the Pan-African crust-forming cycle¹⁷ that includes the ~550-Myr ages reported here (759 ± 5 , 714 ± 70 , 583 ± 25 , 477 ± 30 Myr for H1, CH6 and C5, 6 (ref. 10 respectively). A single rounded grain (CH7) is 98% discordant from 2725 ± 57 Myr toward the 65.5 Myr event, whereas six other mostly rounded grains are totally reset (CH8–11, H6, 7) as if the lead loss was enhanced by extensive pre-impact radiation damage. A single near-concordant 320 ± 31 Myr shocked grain found in Colorado ejecta¹⁰ (C14) and a single 286 ± 14 Myr titanite grain (CH12) from Chicxulub confirm a minor, still younger component at the impact site.

As discrete refractory particles of the target itself, single shocked zircons provide the U–Pb primary age of their individual source even though substantial lead loss occurred during

impact. The remarkable collinearity of the data points for three sites spanning 2,000 km demonstrates not only that each site has an identical (545 Myr) primary age component, but also confirms that post-impact disturbance is absent. This conclusion is also supported by the fact that the six totally reset grains, with the most structural damage, retain a mean lower-intercept age slightly in excess of 65.5 Myr. The link between Colorado ejecta and the Chicxulub crater is particularly significant as impact glass used for Sr and Nd isotopic correlations⁸ is not preserved in North American ejecta outside the Caribbean. Rocks formed 545 ± 5 Myr ago are extremely rare in North America, so the occurrence of shocked zircons of this age in the widespread 65 Myr ejecta deposit and in the Chicxulub crater itself would be difficult or impossible to explain other than by an impact-ejection process. □

Received 2 September; accepted 30 November 1993.

1. Izett, G. A. *Geol. Soc. Am. spec. Pap.* **249**, 1–90 (1990).
2. Sharpton, V. L., Schuraytz, B. C., Burke, K., Murali, A. V. & Ryder, G. *Geol. Soc. Am. spec. Pap.* **247**, 349–357 (1990).
3. Alvarez, W., Alvarez, L. W., Asaro, F. & Michel, H. V. *Geol. Soc. Am. spec. Pap.* **190**, 305–315 (1982).
4. Penfield, G. T. & Camargo, Z. A. in *51st A. Meeting Tech. Prog. Soc. Explor. Geophys. (Abstr.)* 37 (1981).
5. Hildebrand, A. R. et al. *Geology* **19**, 867–871 (1991).
6. Swisher, C. C. et al. *Science* **257**, 954–958 (1992).
7. Sharpton, V. L. et al. *Nature* **359**, 819–821 (1992).
8. Blum, J. D. et al. *Nature* **364**, 325–327 (1993).
9. Bohor, B. F., Betterton, W. J. & Krogh, T. E. *Earth planet. Sci. Lett.* **119**, 419–424 (1993).
10. Krogh, T. E., Kamo, S. L. & Bohor, B. F. *Earth planet. Sci. Lett.* **119**, 425–429 (1993).
11. Hildebrand, A. R. thesis, Univ. Arizona (1992).
12. Lopez Ramos, E. *Geologia de Mexico* 2nd edn Vol. 2 (Univ. Mexico, Mexico City, 1979).
13. Sharpton, V. L. et al. *Science* **261**, 1564–1567 (1993).
14. Krogh, T. E. *Geochim. cosmochim. Acta* **37**, 485–494 (1973).
15. Davis, D. W. *Can. J. Earth Sci.* **19**, 2141–2149 (1982).
16. Stacey, J. S. & Kramers, J. D. *Earth planet. Sci. Lett.* **34**, 207–226 (1975).
17. Krogh, T. E. et al. *Can. J. Earth Sci.* **25**, 442–453 (1988).

ACKNOWLEDGEMENTS. This study was supported by the Natural Sciences and Engineering Research Council (NSERC) and infrastructure grants to T.E.K.

A model for disequilibrium mantle melting incorporating melt transport by porous and channel flows

Hikaru Iwamori

Bullard Laboratories, Department of Earth Sciences, University of Cambridge, Madingley Road, Cambridge CB3 0EZ, UK

ATTEMPTS to understand melt transport in the Earth's mantle have focused on the two mechanisms of porous flow along grain boundaries^{1–4} and channel flow in fractures^{5–10}. To elucidate the contributions of these segregation processes to the chemical evolution of melt and solid, I have developed a disequilibrium melting model for a one-dimensional upwelling mantle in which melt is produced, transported by porous flow and sucked into chemically isolated channels. The model is based on recent disequilibrium melting models^{11,12}, in which the residual melt in the porous flow system need not equilibrate with the residual solid. By changing the parameter values such as the rates of chemical equilibration and melt removal into channels, this model predicts trace element concentrations for various melting conditions, including disequilibrium effects which cannot be assessed quantitatively using previous models for dynamic mantle melting^{1,6,13–15}. Comparison between the model results and compositions of abyssal peridotites¹⁶ supports the idea that, if equilibrium melting is achieved, imperfect

fractional melting (corresponding to 80% melt removal) occurs beneath mid-ocean ridges^{17,18}. If disequilibrium melting is important, however, more efficient melt removal (in other words, chemical isolation from the residue) is required to explain the peridotite data.

The model presented here has been developed from the disequilibrium melting model with porous flow¹² by adding a consideration for melt suction into chemically isolated channels^{6,11}. First, I consider a one-dimensional upwelling column in which melt is produced by adiabatic decompression and percolates upward through the residual solid with continuous chemical equilibration. The rate of chemical equilibration is assumed to be controlled by diffusion of chemical species in the solid. In addition, I consider melt suction from this porous flow system into channels at various depths (Fig. 1). Chemical reactions between the melt transported through channels and surrounding solid are assumed to be negligible. The validity of this assumption will be discussed later. Consequently, concentrations of chemical species in the melt and solid are determined by the balance between melting, advective transport, chemical equilibration and melt suction. The governing equations for a steady state can be expressed as follows:

$$\frac{d}{dz}(\phi \rho_m V_m) = \dot{M} - \dot{S} \quad (1)$$

$$\frac{d}{dz}[(1-\phi)\rho_s V_s] = -\dot{M} \quad (2)$$

$$\frac{d}{dz}(\phi \rho_m V_m C_m) = -\frac{3(1-\phi)}{R} \rho_s D_s \left. \frac{\partial C_s}{\partial r} \right|_{r=R} + \dot{M} C_s|_{r=R} - \dot{S} C_m \quad (3)$$

$$\frac{d}{dz}[(1-\phi)\rho_s V_s \bar{C}_s] = \frac{3(1-\phi)}{R} \rho_s D_s \left. \frac{\partial C_s}{\partial r} \right|_{r=R} - \dot{M} C_s|_{r=R} \quad (4)$$

$$\frac{DC_s}{Dt} = D_s \left(\frac{\partial^2 C_s}{\partial r^2} + \frac{2}{r} \frac{\partial C_s}{\partial r} \right) \quad (5)$$

where z is the vertical co-ordinate, ϕ is the volume fraction of melt in the porous flow system, ρ_m and ρ_s are the densities of melt and solid, V_m and V_s are the upward velocities of melt and solid in the porous flow system, C_m and C_s are the concentrations by weight of a chemical species in melt and solid, $\bar{C}_s$ is the average concentration of a chemical species in a solid grain, r is the radial coordinate within a solid grain, R is the radius of a solid grain, D_s is the diffusion coefficient of a chemical species in solid, $\dot{M}$ is the melting rate per unit volume and $\dot{S}$ is the rate of melt suction per unit volume from the porous flow system into channels. The solid grain is assumed to be a sphere with radius R .

Equations (1) and (2) represent mass conservation for the melt and solid. Equations (3) and (4) represent mass conservation of a chemical species in the melt and solid, where diffusion of a chemical species in the z -direction has been neglected¹⁹. The first term on the right-hand side of equations (3) and (4) describes mass flux through the interface, which is controlled by diffusion in the solid. I assume that a solid grain melts from the rim ($r=R$), which is expressed as the second term on the right-hand side of equations (3) and (4). The last term of equations (1) and (3) is added to the equations for the model of ref. 12, and expresses the melt suction into channels. Equation (5) expresses diffusion of a chemical species within a solid grain in the r -direction, using the lagrangian derivative. In addition to equations (1)–(5), simplified equations for conservation of number of grains, momentum and energy are employed as in ref. 12. Darcy's law is used to describe the differential flow between melt and solid in the porous flow system. The melting rate $\dot{M}$ is calculated from conservation of energy, assuming that the melting proceeds linearly with depth by adiabatic decompression^{12,15}.

It should be stressed that the equations described above govern conservation of mass, momentum and energy only in the porous flow system. Once the melt is extracted into a chemically isolated set of channels, neither the flow nor the chemical interaction with the porous flow system is calculated. In this regard, the model is similar to previous melting models with melt suction^{6,11}. Although the previous models do not account for the porous flow, both models assume that a residual melt fraction is retained and the rest of the melt is removed from the system. In the present model $\dot{S}$ controls the amount of melt removal from the system, and is likely to be a function of many factors such as the pressure difference of melt between the inside of channels and the surroundings, the spacing of channels and so on^{8,9}. Here I assume simply that $\dot{S}$ is proportional to $\dot{M}$, that is $\dot{S} = S\dot{M}$ where S is a constant corresponding to the melt fraction that is removed from the porous flow system. As in the previous melting models with melt suction^{6,11}, if $S=0$ all the melt remains in the porous flow system and can re-equilibrate with the residual solid to produce batch melts^{12,13,15,20}. If $S=1$ all the melt is removed instantaneously (that is $\phi=0$ at all z), which is identical to fractional melting.

The principal difference between this and previous models for dynamic mantle melting^{1,6,13–15} is that the residual melt left in the porous flow system need not equilibrate with the residual solid during melting. As described in the recent disequilibrium melting models^{11,12}, chemical equilibrium is only enforced between the residual melt and the contact surface of the solid grains. The bulk partition coefficients of the entire solid may change along the flow due to a phase transition (for example,

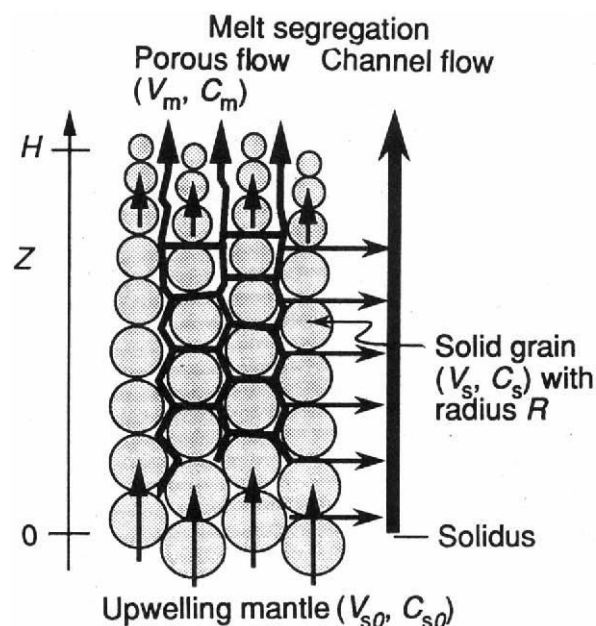


FIG. 1 A model for one-dimensional mantle melting associated with melt transport by porous and channel flows. Unmelted mantle material ascends at constant velocity V_{s0} with concentration C_{s0} for a chemical species and begins melting at depth $z=0$. H is the height of the melting column. The melt produced with concentration C_m for the chemical species percolates upwards at velocity V_m through the residual solid grains with velocity V_s and concentration C_s . In addition to this porous flow system, melt suction into chemically isolated channels can occur at various depths as is shown by horizontal arrows. Consequently, the residual melt fraction in the porous flow system is controlled by both efficiency of melt percolation (parameter Q of ref. 12) and melt suction (parameter S of the present model). Once melt is sucked into channels, neither the flow nor the chemical interaction is calculated in the present model. Such melt is simply assumed to ascend to the surface instantaneously, as in the previous melt suction models^{6,11}.

garnet peridotite to spinel peridotite) or by non-modal melting, which is incorporated in the present model. This model also assumes that solid grains have no compositional zoning before melting starts.

The governing equations are rearranged and non-dimensionalized with a Boussinesq-type approximation. Solutions to the governing equations are obtained when four dimensionless parameters are specified. These parameters are $\phi_0 = MH/\rho_{s0}V_{s0}$, $Q = k_0\Delta\rho g/V_{s0}$, $\tau = R_0^2V_{s0}/D_sH$ and $S = \dot{S}/\dot{M}$, where H is the height of the melting column, ρ_{s0} is the density of solid before melting starts, V_{s0} is the upwelling velocity of solid before melting starts, k_0 is a parameter which controls the permeability and depends on R and the viscosity of melt, $\Delta\rho$ is the density difference between melt and solid, g is the acceleration due to gravity, R_0 is the radius of solid grains before melting starts and ϕ_0 represents the total degree of melting at $z=H$. Q measures the efficiency of melt percolation in the porous flow

system (0 means no percolation, and ∞ means instantaneous percolation). τ represents the characteristic time for local chemical equilibration (0 means instantaneous equilibration, and ∞ means no equilibration). If we replace the radius of the solid grain, R_0 , with the distance between the channels, d , then τ represents the characteristic time for chemical reaction between the melt inside channels and surrounding rocks. For realistic melting situations, $d \gg 0.1$ m results in $\tau \gg 1$, which corresponds to the condition for negligible reaction between the melt inside channels and the surrounding rocks^{20,21}. This condition is not affected by the velocity of melt in channels, and is likely to be satisfied for dykes observed in peridotites^{7,10}. The required condition, $d \gg 0.1$ m (sparse distribution of channels to be chemically isolated), however does not imply a mechanism for formation of the channels, such as fracturing^{5,7} or localization of melt paths in a deforming partially molten rock due to fluid dynamical instability²². S represents the efficiency of melt suction.

The numerical calculations were carried out for the range of $\phi_0 = 0.1 \rightarrow 1$, $Q = 0 \rightarrow \infty$, $\tau = 0 \rightarrow 10^3$ and $S = 0 \rightarrow 1$ to simulate extreme as well as realistic conditions. By changing the parameter values, this model is able to describe chemical variations for various melting conditions, including the melting models proposed so far. Figure 2 shows two examples for equilibrium batch-fractional melting, $(Q, \tau, S) = (0, 0, 0 \rightarrow 1)$ (Fig. 2a), and equilibrium-disequilibrium fractional melting, $(0, 0 \rightarrow 1, 1)$ (Fig. 2b). An increase of S causes depletion of incompatible elements in the residual solid (for example Ce in Fig. 2a), due to effective melt suction which prevents the residual solid from reacting with melt. Fractional melting occurs when $S=1$ (complete melt suction) and causes the largest depletion. Whereas S controls the amount of melt available to equilibrate with the solid, which is analogous to the residual melt fraction in the previous melting models^{6,11}, τ controls the degree of local equilibration of the residual melt and solid. An increase of τ causes enrichment of the residual solid (Fig. 2b), because incompatible elements are not fully extracted due to slow diffusion. The estimation of disequilibrium effects is important, especially when S is large, because rapid melt separation from the residual solid considerably limits the time for chemical equilibration. Aluminium, Ca, Sr and Sm (and probably other light rare-earth elements) have low diffusivity in diopside^{23,24} (10^{-19} m² s⁻¹ at 1,200 °C) and have τ of 0.2–20, assuming $R_0 = 10^{-3}$ – 10^{-2} m, $H = 5 \times 10^4$ m and $V_{s0} = 10^{-9}$ m s⁻¹ which correspond to melting conditions beneath mid-ocean ridges. These elements may be partitioned in disequilibrium. This possibility will be discussed later.

Of the three dimensionless parameters Q , τ and S , Q has insignificant effects, because the melt and solid compositions are essentially independent of the relative flow between melt and solid in one-dimensional steady-state systems^{12,13,15,20}. In two- or three-dimensional problems in which melt porous flow diverges from the solid flow, Q may affect the composition of each phase as much as S . This effect is neglected in the present model, which restricts applications of the model to one-dimensional cases (for example, axial parts of ridges or plumes). On the other hand, Q affects the composition of residual bulk (residual melt plus solid) through a change in the residual melt fraction even in one-dimensional situations¹². Within the realistic range of Q (10^{-3} – 10^5) (refs 12, 15), however, this effect is also negligible. Further, the melt composition is relatively insensitive to S and τ , when the melt is accumulated. Therefore I concentrate on the effects of S and τ on the residual solid and bulk compositions in the following application.

Based on the model predictions, I assess the melting processes beneath the America–Antarctic and Southwest Indian Ridge systems. The trace-element data of peridotites dredged from these ridges¹⁶ are used. These peridotites are thought to be the residues of variable degrees of one-dimensional adiabatic melting, the degree of which depends on the distance from hotspots¹⁶. I calculated the bulk concentrations of trace elements in the residual peridotites using the clinopyroxene compositions, modal compo-

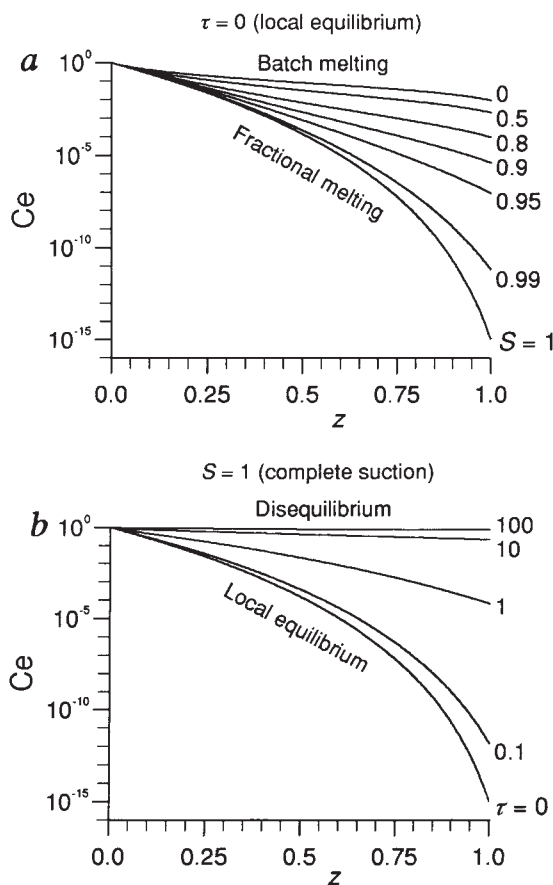
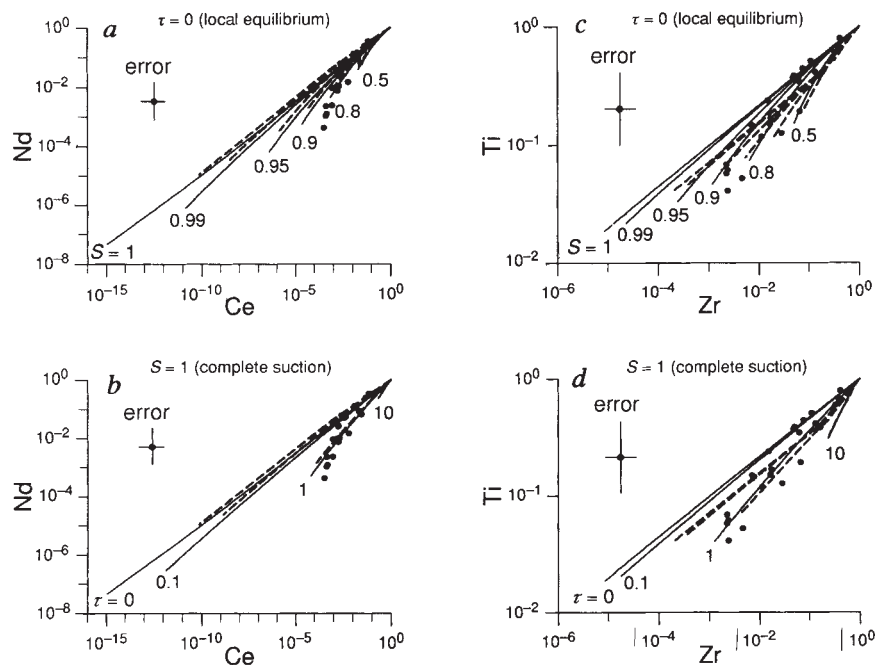


FIG. 2 Ce concentrations in the residual solid ($\bar{C}_s$) for various melting conditions along the flow (z -direction), with $\phi_0 = 0.25$ (up to 25% degree of melting) and $Q = 0$ (no porous flow). The concentrations are normalized to C_{s0} . The bulk partition coefficient K for non-modal melting in the spinel peridotite field is calculated based on Shaw's equation³², $K = (D^0 - PF)/(1 - F)$ where D^0 is the initial bulk partition coefficient of a chemical species for the mineral assemblage before melting starts and P is the partition coefficient of a chemical species for the mineral assemblage entering the melt. F is the degree of melting. D^0 and P for Ce are calculated using the partition coefficient data from ref. 26 and phase proportions in the source and the melt modes from Table A2 of ref. 16 ($D_{Ce}^0 = 0.019$ and $P_{Ce} = 0.067$). a, Ce concentrations for $\tau = 0$ (local equilibrium condition) and various values of S from 0 to 1. $S = 0$ results in no melt suction into channels, and corresponds to batch melting. $S = 1$ results in complete melt suction into channels, and corresponds to fractional melting. b, Ce concentration for $S = 1$ and various values of τ from 0 to 10^2 . $\tau = 0$ indicates local equilibrium, whereas almost no equilibration occurs with $\tau = 10^2$.

FIG. 3 Trace-element variations in the residual bulk (residual melt plus solid) for various melting conditions with up to 25% degree of melting ($\phi_0=0.25$) and $Q=10^4$. Solid circles are the calculated bulk concentrations in the dredged peridotites¹⁶ (see text). The concentrations are normalized to a depleted mantle composition^{16,17}. The errors involved in the calculated bulk compositions are estimated based on the errors involved in the modal and the chemical analyses of abyssal peridotites (refs 16, 33 and K. T. M. Johnson, personal communication). a. Variations of Ce with Nd for $\tau=0$ (local equilibrium condition) and various S . b. Variations of Ce with Nd for $S=1$ (complete melt suction) and various τ . c. Variations of Zr with Ti for $\tau=0$ (local equilibrium condition) and various S . d. Variations of Zr with Ti for $S=1$ (complete melt suction) and various τ . Two sets of partition coefficient data for the spinel and garnet peridotite assemblages^{25,26} were used, with phase proportions in the source and the melt modes from Table A2 of ref. 16. Solid lines correspond to 25% melting in the spinel peridotite field ($D_{\text{Ce}}^0=0.019$, $P_{\text{Ce}}=0.067$, $D_{\text{Nd}}^0=0.036$, $P_{\text{Nd}}=0.13$, $D_{\text{Zr}}^0=0.032$, $P_{\text{Zr}}=0.076$, $D_{\text{Ti}}^0=0.091$ and $P_{\text{Ti}}=0.23$). Dotted lines correspond to 10% melting in the garnet peridotite field ($D_{\text{Ce}}^0=0.016$, $P_{\text{Ce}}=0.046$, $D_{\text{Nd}}^0=0.036$, $P_{\text{Nd}}=0.11$, $D_{\text{Zr}}^0=0.059$, $P_{\text{Zr}}=0.20$, $D_{\text{Ti}}^0=0.11$ and $P_{\text{Ti}}=0.29$) and additional 15% melting in the spinel peridotite field ($D_{\text{Ce}}^0=0.015$, $P_{\text{Ce}}=0.067$, $D_{\text{Nd}}^0=0.029$, $P_{\text{Nd}}=0.13$, $D_{\text{Zr}}^0=0.029$, $P_{\text{Zr}}=0.076$, $D_{\text{Ti}}^0=0.081$ and $P_{\text{Ti}}=0.23$). Inclusion of



10% melting in the garnet peridotite field makes the fit for the Zr–Ti data worse, which suggests that the extent of melting in the garnet peridotite field is small (<10%) beneath the ridges.

sitions of the peridotites and partition coefficients between the minerals^{16,25,26}. These concentrations are normalized to a depleted mantle composition^{16,17}, and plotted in Fig. 3 together with predicted residual bulk compositions for various melting conditions.

Figure 3a and c show predicted concentrations of Ce–Nd and Zr–Ti under local equilibrium conditions ($\tau=0$) for up to 25% degree of melting. Obviously we cannot explain the peridotite compositions by perfect fractional melting ($S=1$) in terms of both the degree of depletion and the ratio between the elements. As S decreases the degree of depletion decreases, and the ratios Ce/Nd and Zr/Ti increase. If we take a value of S of ~ 0.8 , the predicted variation can account for the peridotite variation, although a degree of melting slightly greater than 25% is required to explain the depleted end. This result is essentially identical to the previous work on the abyssal peridotites, which imply imperfect fractional melting beneath the ridges^{17,18}. The result is also consistent with the compositions of ridge basalts^{6,27–30} and depleted melt inclusions in olivine³¹, which suggest that the basalts are formed by sampling of near-fractional melts from various depths.

As discussed previously, τ for Ce and Nd is estimated to be >0.2 for a melting condition beneath mid-ocean ridges. Considering that even $\tau=0.1$ causes some degree of disequilibrium for a high value of S (Fig. 2b), local equilibrium may have been violated beneath the ridges. Disequilibrium melting increases incompatible element concentrations in the solid, in opposition to fractional melt suction which removes material that is available to enrich the solid (Fig. 2). Thus if disequilibrium melting is important, the more efficient removal of melt from the residue is required to explain the peridotite compositions. Figure 3b and d present an endmember model with $S=1$ (complete melt suction), which shows that the peridotite data can be explained also by disequilibrium melting with $\tau=1$. The true condition probably exists between $(\tau, S)=(0, 0.8)$ and $(1, 1)$. But we cannot precisely determine how much disequilibrium is significant, because the estimation of τ strongly depends on R_0 which is not

well constrained. In any case a high value of S (>0.8) is required for melting beneath the ridges (that is more than 80% of the melt produced must be chemically isolated from the residue). To test these equilibrium–disequilibrium melting models, we need further studies on physical aspects of melt segregation processes including effective melt removal from the porous flow system into chemically isolated channels. □

Received 4 December 1992; accepted 10 November 1993.

- McKenzie, D. J. *Petrol.* **25**, 713–765 (1984).
- Richter, F. M. & McKenzie, D. J. *Geol.* **92**, 729–740 (1984).
- Ribe, N. M. *Geophys. J. R. astr. Soc.* **83**, 487–501 (1985).
- Scott, D. R. & Stevenson, D. J. *J. geophys. Res.* **91**, 9283–9296 (1986).
- Nicholas, A. & Jackson, M. J. *Petrol.* **23**, 568–582 (1982).
- McKenzie, D. *Earth planet. Sci. Lett.* **72**, 149–157 (1985).
- Nicholas, A. *J. Petrol.* **27**, 999–1022 (1986).
- Ribe, N. M. *Geophys. Res. Lett.* **13**, 1462–1465 (1986).
- Sleep, N. H. *J. geophys. Res.* **93**, 10255–10272 (1988).
- Takahashi, N. *Nature* **359**, 52–55 (1992).
- Qin, Z. *Earth planet. Sci. Lett.* **112**, 75–90 (1992).
- Iwamori, H. *Earth planet. Sci. Lett.* **114**, 301–313 (1993).
- Ribe, N. M. *Earth planet. Sci. Lett.* **73**, 361–376 (1985).
- Richter, F. M. *Earth planet. Sci. Lett.* **77**, 333–344 (1986).
- Ribe, N. M. *Earth planet. Sci. Lett.* **88**, 37–46 (1988).
- Johnson, K. T. M., Dick, H. J. B. & Shimizu, N. *J. geophys. Res.* **95**, 2661–2678 (1990).
- Johnson, K. T. M. & Dick, H. J. B. *J. geophys. Res.* **97**, 9219–9241 (1992).
- Elthon, D. J. *Geophys. Res.* **97**, 9015–9025 (1992).
- Navon, O. & Stolper, E. *J. Geol.* **95**, 285–307 (1987).
- Iwamori, H. *Geophys. Res. Lett.* **19**, 309–312 (1992).
- Spiegelman, M. & Kenyon, P. *Earth planet. Sci. Lett.* **109**, 611–620 (1992).
- Stevenson, D. J. *Geophys. Res. Lett.* **16**, 1067–1070 (1989).
- Freer, R., Carpenter, M. A., Long, J. V. P. & Reed, S. J. B. *Earth planet. Sci. Lett.* **58**, 285–292 (1982).
- Sneeringer, M., Hart, S. R. & Shimizu, N. *Geochim. cosmochim. Acta* **48**, 1589–1608 (1984).
- Pearce, J. A. & Norry, M. J. *Contr. Miner. Petrol.* **69**, 33–47 (1979).
- Hanson, G. N. A. *Rev. Earth planet. Sci.* **8**, 371–406 (1980).
- Klein, E. M. & Langmuir, C. H. *J. geophys. Res.* **92**, 8089–8115 (1987).
- McKenzie, D. & Bickle, M. J. *J. Petrol.* **29**, 625–679 (1988).
- Salter, V. J. M. & Hart, S. R. *Nature* **342**, 420–422 (1989).
- Elliott, T. R., Hawkesworth, C. J. & Grönvold, K. *Nature* **351**, 201–206 (1991).
- Sobolev, A. V. & Shimizu, N. *Nature* **363**, 151–154 (1993).
- Shaw, D. M. *Geochim. cosmochim. Acta* **34**, 237–243 (1970).
- Dick, H. J. B., Fisher, R. L. & Bryan, W. B. *Earth planet. Sci. Lett.* **69**, 88–106 (1984).

ACKNOWLEDGEMENTS. The author thanks P. Beattie, K. Johnson, H. Kerr, D. McKenzie, P. Morgan, C. Richardson and T. Tanaka for discussions and help, and D. Scott for an extensive review. This work was supported by the JSPS.

Modelling territoriality and wolf–deer interactions

M. A. Lewis* & J. D. Murray†

* Department of Mathematics, University of Utah, JWB 233, Salt Lake City, Utah 84112, USA

† Department of Applied Mathematics, University of Washington, Seattle, Washington 98195, USA

WE propose here a mechanism for territorial pattern formation in wolves (*Canis lupus*) and analyse it using a spatially explicit mathematical model incorporating wolf movement and scent marking. Model results reflect field observations from northeastern Minnesota: buffer zones where wolves are scarce arise between adjacent packs, and near these buffer zones there are increased levels of scent marking^{1,2}. Territories are arranged in a spatial mosaic (Fig. 1) which covers the wolf range. In the model formulation no assumptions are made about actual existence or extent of wolf territory and buffer zones; these arise naturally as stable steady-state solutions to the equations. We show mathematically how reduced predation by wolves in the buffer zones provides a refuge for prey species. Field studies support this; distribution of a primary prey species, white-tailed deer (*Odocoileus virginianus*), correlates negatively with that of wolves. Deer are found primarily in buffer zones^{3,4} (Fig. 2).

Elaborate spatial patterns of wolf scent marks⁵ are used to advertise precise information about territorial claims⁶. Behavioural studies indicate that it is the raised leg urination (RLU) which plays the key role in scent marking^{5,7}. Out of a typical pack of 5–15 wolves⁸, a few mature dominant members leave RLUs regularly on trails; a travelling wolf may encounter one RLU every two to three minutes⁵. Marking frequency roughly doubles near territorial borders, giving rise to 'bowl-shaped' distributions of RLUs across territories, with the raised edges of the olfactory bowl located at the territorial boundaries⁵. Observations indicate scrutiny of RLUs⁷ and some aversion to foreign RLUs made by neighbouring packs⁵.

We propose a mechanism for generating the territorial patterns using a mechanistic, spatially explicit mathematical model with simple rules for scent marking and movement. Making no underlying assumptions about the size and extent of the wolf territories themselves, we show how the territorial patterns actually arise naturally as stable steady-state solutions to the equations.

Our key modelling assumption is that wolf movement and behaviour are mediated by the absence/presence of foreign RLUs. In the absence of foreign RLUs: (1) movement is primarily dispersive as individuals search for food and other resources, and (2) RLU-marking occurs at low levels. The presence of foreign RLUs elicits two responses: (1) movement towards an organizing centre for the pack such as a den, above-ground rendezvous site or familiar location⁹; and (2) increase in RLU-marking frequency⁵. The RLU marks are assumed to lose intensity with age.

The expected location of an RLU-marking wolf is described by a probability density function denoting the chance of finding it at point x and time t . For any given pack, these are summed over the number of RLU-marking wolves to yield the expected density of RLU-marking wolves, henceforth referred to as the expected density of wolves in a pack. For a model of two adjacent interacting wolf packs, pertinent state variables are expected densities of wolves in packs 1 and 2 ($u(x, t)$ and $v(x, t)$); and of RLUs from packs 1 and 2 ($p(x, t)$ and $q(x, t)$).

Components of the wolf movement are expressed mathematically by:

$$\frac{\partial u}{\partial t} = -\nabla \cdot \{J_{du} + J_{cu}\} \quad (1)$$

and

$$\frac{\partial v}{\partial t} = -\nabla \cdot \{J_{dv} + J_{cv}\} \quad (2)$$

where J_{du} and J_{dv} describe dispersive movement via diffusion and J_{cu} and J_{cv} describe movement towards organizing centres situated at x_u and x_v by convection towards x_u and x_v , respectively.

Specific forms for the fluxes in equations (1) and (2) were derived under the assumptions that dispersive movement is modelled by simple diffusion ($J_{du} = -d\nabla u$, $J_{dv} = -d\nabla v$) with diffusion coefficient $d > 0$; and that convective movement is at a rate proportional to the expected density of foreign RLU

$$(J_{cu} = -cuq(x - x_u)/|x - x_u|, J_{cv} = -cvp(x - x_v)/|x - x_v|)$$

with convective proportionality constant $c > 0$.

Equations for RLU-marking and decay are:

$$\frac{\partial p}{\partial t} = u(l + mq) - fp \quad (3)$$

and

$$\frac{\partial q}{\partial t} = v(l + mp) - fq \quad (4)$$

where $l > 0$ denotes low-level continual RLU marking, $m > 0$ describes increased RLU-marking levels in the presence of foreign RLUs, and $f > 0$ represents first-order RLUs decay kinetics.

Analysis indicates the existence of a stable, spatially heterogeneous steady-state solution to equations (1) to (4). Describing territorial patterns and corresponding levels of the spatially distributed RLUs, this solution (Fig. 3) can be found analytically by integrating steady-state versions of equations (1) to (4) subject to zero-flux boundary conditions for u and v . The solution predicts the partitioning of available space into territories and the formation of territorial boundaries near which there are low levels of expected wolf density and high levels of expected RLU density (Fig. 3). This clearly reflects the field observations regarding territorial patterns, interpack buffer zones and bowl-shaped RLU densities. Thus, the simple rules for scent marking and movement given here are sufficient to explain territorial patterns.

Here, scent marks are crucial to the formation of territories; if scent marking is not included ($l, m = 0$ in equations (3) and (4)) then $p, q \rightarrow 0$ and equations (1) and (2) are reduced to diffusion equations so that the territories do not form. It can also be shown that a necessary condition for the bowl-shaped

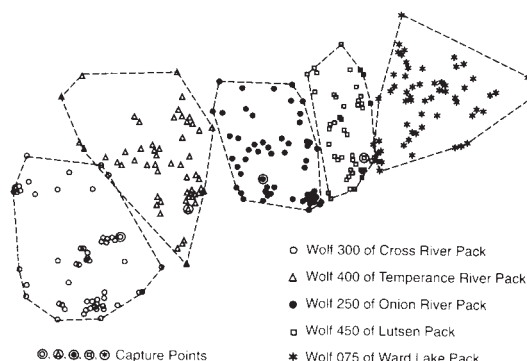


FIG. 1 Radiolocations and home range boundaries of five adult and yearling timber wolves radiotracked by aircraft, 27 May–22 October 1971 in northeastern Minnesota. Redrawn from Fig. 4 of ref. 2 with permission.

RLU patterns with interior maxima for p and q (Fig. 3) is that foreign RLUs elicit a sufficiently large increase in the RLU-marking rate. Mathematically, this condition is met when m exceeds a critical value.

We now consider an extension of the above model which can be used to predict the negative correlation between wolf territories and the white-tailed deer distribution (Fig. 2). For the purposes of illustration, we assume: (1) wolf territories are stationary and stable (Fig. 3); (2) Holling type 1 functional response of deer to wolf predation; (3) Beverton–Holt density-dependent population dynamics for the deer in the absence of wolf predation. Denoting $h(x, t)$ as the expected density of deer, we have

$$\frac{\partial h}{\partial t} = -\psi(u(x) + v(x))h \quad (5)$$

where $u(x)$ and $v(x)$ denote the expected densities of pack 1 and pack 2 wolves, ψ is the predation rate and $0 \leq t \leq T$, the year being T time units long. Steady-state solutions, yielding constant values when deer densities are compared from one spring to the next, satisfy the Beverton–Holt dynamics:

$$h(0, x) = \frac{\lambda h(T, x)}{1 + (\lambda - 1)h(T, x)/K} \quad (6)$$

where $\lambda > 1$ and K measure the population growth rate and carrying capacity of the deer in the absence of predation, respectively. Solving equation (5) subject to the condition of equation (6) gives steady-state solutions as

$$h(0, x) = \max \{0, K(\lambda - \exp \{[u(x) + v(x)]\psi T\})/(\lambda - 1)\} \quad (7)$$

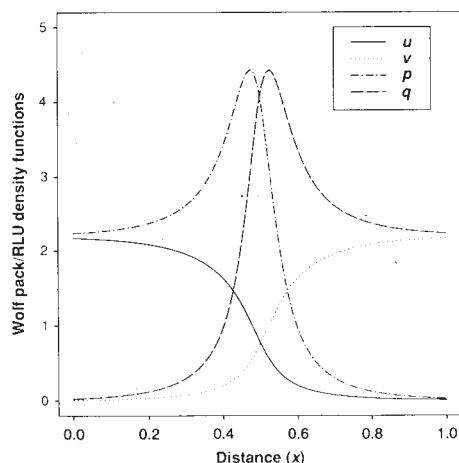


FIG. 3 Territorial patterns arise naturally as stable steady-state solutions to the model. Shown is a stable steady-state solution for equations (1) to (4) for the case $l=1.0$, $f=1.0$, $m=1.1$,

$$J_{c_u} = -uq(x - x_u)/|x - x_u|, \quad J_{c_v} = -vp(x - x_v)/|x - x_v| \\ J_{d_u} = -0.3\nabla u, \quad J_{d_v} = -0.3\nabla v$$

$x_u=0$ and $x_v=1$. A territorial boundary is found at the point $x=0.5$. Near this boundary the expected wolf-densities, $u(x)$ and $v(x)$, are low and expected RLU densities, $p(x)$ and $q(x)$, are high. Numerical solution used the method of lines and Gear's method.

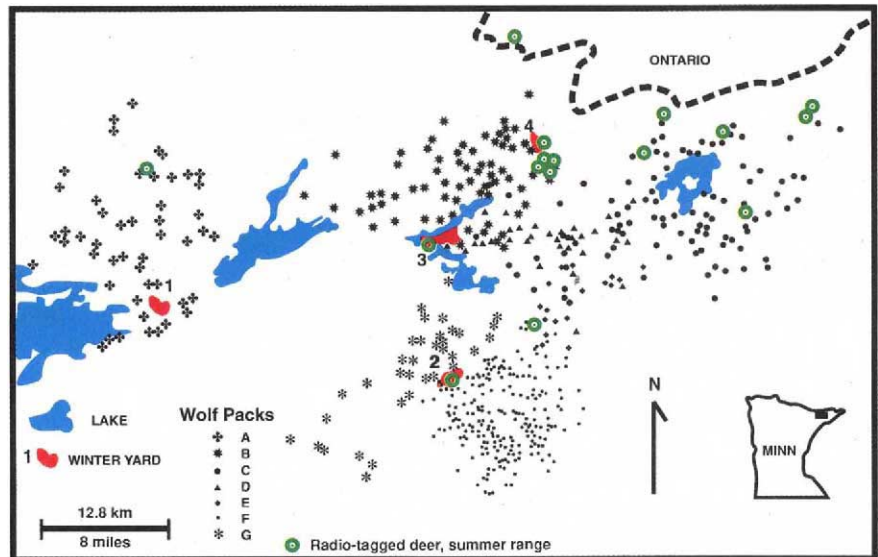


FIG. 2 Winter yards and summer home ranges of radio-tagged deer in relation to wolf-pack territories. Wolf-pack territories: A, Glenmore L. pack; B, Newton L. pack; C, Ensign L. pack; D, Greenstone L. pack; E, Pagami L. pack; F, Harris L. pack; G, Birch L. pack in northeastern Minnesota. Redrawn from Fig. 4 in Hoskinson and Mech³ with permission. (Roads have been removed and colour has been added.)

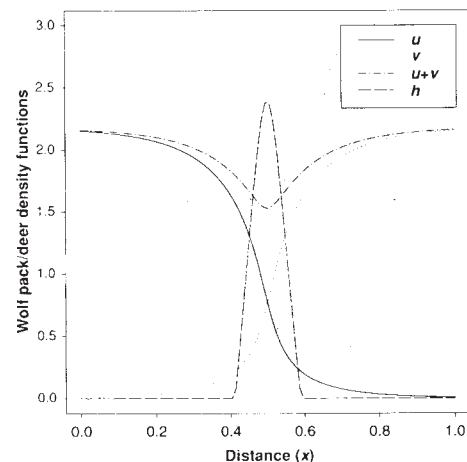


FIG. 4 Deer are found primarily in the narrow corridors between the territories of adjacent wolf packs. Shown is the steady-state distribution of deer in the spring given by equation (7) and $u(x)$ and $v(x)$ given as in Fig. 3, and $\psi T=0.3$, $\lambda=2.0$ and $K=5.0$.

Thus deer are found primarily where the combined wolf densities are lowest, in the buffer zones between packs (Fig. 4).

As noted by Mech¹⁰, evidence for a similar situation involving deer and human societies is given by Hickerson's study of Sioux and Chippewa Indians in 1780–1850¹¹. Warfare between the two tribes gave rise to a buffer zone which was normally unoccupied. In turn, reduced hunting pressure in this buffer zone effectively provided a refuge for the deer. But when a long truce was maintained, the buffer zone was destroyed and famine ensued. □

Received 9 August; accepted 18 October 1993.

1. Mech, L. D. Technical report, US Forest Service Research Paper NC-97 (North Central Forest Experimental Station, St Paul, Minnesota, 1973).
2. Van Ballenberghe, V., Erickson, A. W. & Byman, D. *Wildl. Mono.* **43**, 1–43 (1975).

3. Hoskinson, R. L. & Mech, L. D. *J. Wildl. Manage.* **40**, 429–441 (1976).
4. Rogers, L. L., Mech, L. D., Deanna, K. D., Peek, J. & Korb, M. *J. Wildl. Manage.* **44**, 253–258 (1980).
5. Peters, R. P. & Mech, L. D. *Am. Sci.* **63**, 628–637 (1975).
6. Mech, D. L. *The Way of The Wolf* (Voyager, Stillwater, Minnesota, 1991).
7. Merti-Millhollen, A. S., Goodman, P. A. & Klinghamer, E. *Zoo Biol.* **5**, 7–20 (1986).
8. Mech, L. D. *The Wolf: The Ecology and Behaviour of an Endangered Species* (Natural History Press, Garden City, New York, 1970).
9. Peters, R. in *The Behaviour and Ecology of Wolves* (ed. Klinghamer, E.) 119–152 (Garland, New York, 1979).
10. Mech, L. D. *Science* **198**, 320–321 (1977).
11. Hickerson, H. in *Man, Culture and Animals: The Role of Animals in Human Ecological Adjustments* (eds Leeds, A. & Vayd, A.) 43–65 (American Association for the Advancement of Science, Washington DC, 1965).

ACKNOWLEDGEMENTS. We thank P. Karieva and D. Mech for commenting on earlier drafts. This work was supported in part by grants from the United States National Science Foundation, Division of Mathematical Sciences.

Development of obesity in transgenic mice after genetic ablation of brown adipose tissue

Bradford B. Lowell*, Vedrana S-Susulic*, Andreas Hamann*, Joel A. Lawitts†, Jean Himms-Hagen‡, Bert B. Boyer§, Leslie P. Kozak§ & Jeffrey S. Flier*

* Charles A. Dana Research Institute and Harvard-Thorndike Laboratory of Beth Israel Hospital, Department of Medicine and † Department of Pathology, Beth Israel Hospital and Harvard Medical School, Boston, Massachusetts 02215, USA

‡ Department of Biochemistry, Faculty of Medicine, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada

§ Jackson Laboratory, 600 Main Street, Bar Harbor, Maine 04609, USA

BROWN adipose tissue, because of its capacity for uncoupled mitochondrial respiration^{1,2}, has been implicated as an important site of facultative energy expenditure^{3–5}. This has led to speculation that this tissue normally functions to prevent obesity^{3–5}. Attempts to ablate or denervate brown adipose tissue surgically have been uninformative because it exists in diffuse depots and has substantial capacity for regeneration and hypertrophy⁶. Here we have used a transgenic toxigenic approach^{7,8} to create two lines of transgenic mice with primary deficiency of brown adipose tissue. At 16 days, both lines have decreased brown fat and obesity. In one line, brown fat subsequently regenerates and obesity resolves. In the other line, the deficiency persists and obesity, with its morbid complications, advances. Obesity develops in the absence of hyperphagia, indicating that brown fat deficient mice have increased metabolic efficiency. As obesity progresses, transgenic animals develop hyperphagia. This study supports a critical role for brown adipose tissue in the nutritional homeostasis of mice.

Regulatory elements of the gene for the uncoupling protein (UCP), a specific protein marker for brown adipose tissue (BAT)^{2,9}, were used to drive expression of diphtheria toxin A-chain (UCP-DTA) or an attenuated mutant (UCP-176) (Fig. 1a). Two lines of mice were created, UCP-DTA and UCP-176, both of which have ablated BAT and obesity (Fig. 1). To quantify the degree of BAT ablation, immunoblotting with UCP antisera was used to assess the interscapular BAT depot content of UCP. The interscapular depot is well circumscribed and represents the largest single collection of BAT in mice⁵. Interscapular UCP content was decreased modestly in UCP-DTA mice and markedly in UCP-176 mice (Fig. 1b,c). In 16-day-old UCP-DTA transgenic mice, UCP content was decreased by 68% whereas in

UCP-176 transgenic mice it was reduced by 96%. One difference between the UCP-DTA and UCP-176 lines is that the former has microphthalmia and cataracts but the latter does not; this suggests that the eye abnormality is related to the site of transgene integration and is unrelated to the ablation of BAT.

Total body lipid in 16-day-old mice was increased by 31% in UCP-DTA mice and by 68% in UCP-176 mice (Fig. 1d), thus

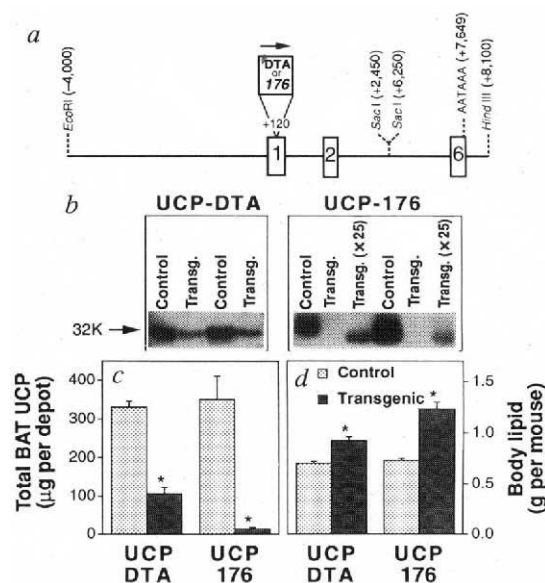


FIG. 1 a, UCP-DTA and UCP-176 transgenes. UCP-1 is a UCP minigene which has previously been shown in transgenic mice to be expressed exclusively in BAT²³. The genetic ablation vectors were constructed to include all of UCP-1 plus an additional 850 base pairs of 5'-distal UCP sequence. Full-strength DTA²⁴ or a 50-fold less toxic, attenuated mutant termed 176 (refs 25, 26), were inserted into the first exon of the UCP minigene at position +120 to create UCP-DTA and UCP-176, respectively. The entire vector was excised from the plasmid, gel purified and injected into the pronuclei of fertilized FVB/N mouse embryos (Taconic Labs, Gormantown, New York). Care of mice was in accordance with institutional guidelines. Numbers shown are relative to the UCP transcription start site and the arrow represents the orientation of DTA and 176 coding sequence. b–d, Sixteen-day-old UCP-DTA and UCP-176 mice. Heterozygous transgenic mice (Transg.) and their nontransgenic littermates (Control) were analysed at age 16 days, before weaning (UCP-DTA: control = 12 (7 female, 5 male), transgenic = 13 (7 female, 6 male); UCP-176: control = 8 (5 female, 3 male), transgenic = 7 (4 female, 3 male). Body weight of transgenic mice at age 16 days was not significantly different from controls (mean $\pm$ s.e. = 7.3 ± 0.2 g). b, Immunoblotting of BAT homogenates for UCP. Each lane contains 1/2,000th of the total depot protein content except for lanes labelled (x25) where 25 times that amount was loaded. c, Quantification of UCP. Immunoblots of BAT interscapular homogenates were prepared with 5 μ g of homogenate protein per lane and purified mouse UCP standards (25–1,000 ng) and analysed with a phosphor-imager. Total interscapular BAT depot UCP was determined by adjusting for total interscapular depot protein content. d, Total body lipid content. Lipid content was assessed using alcoholic potassium hydroxide digestion with saponification of all fats, neutralization and then enzymatic determination of glycerol^{22,27}. Body lipid was determined by multiplying mols of glycerol per mouse and the average relative molecular mass of a triglyceride molecule (860 g mol⁻¹). All values are shown as mean $\pm$ s.e. (* $P < 0.01$).

METHODS. BAT was homogenized as previously described²⁸. Homogenate protein and purified mouse UCP standards were added to a 2% SDS loading buffer and then separated by SDS–polyacrylamide gel electrophoresis (10% polyacrylamide). The proteins were transferred to nitrocellulose, blocked in 5% milk and probed with rabbit anti-hamster UCP antisera diluted 1:200. The bound antibody was detected with ¹²⁵I-labelled goat anti-rabbit IgG (NEN-Dupont). The blots were analysed using a phosphor-imager equipped with Image Quant software (Molecular Dynamics, Benton, New Jersey).

Present address: Institute of Arctic Biology, University of Alaska Fairbanks, Fairbanks, Alaska 99775, USA.

TABLE 1 Summary of data obtained on older UCP-DTA mice

	Total BAT UCP (μg per depot)	Body weight (g)	Total body lipid (g)	Food intake (g per week)	Serum analyses			
					Glucose (mM)	Insulin (pM)	Triglyceride (mg dl ⁻¹)	Cholesterol (mg dl ⁻¹)
Male (8 weeks)								
Control (n=5)	246 $\pm$ 21	25 $\pm$ 1	2.3 $\pm$ 0.2	33 $\pm$ 1	15 $\pm$ 1	380 $\pm$ 90	42 $\pm$ 3	101 $\pm$ 12
Transgenic (n=5)	32 $\pm$ 2*	39 $\pm$ 1*	8.8 $\pm$ 0.9*	53 $\pm$ 2*	24 $\pm$ 2*	1,910 $\pm$ 800	60 $\pm$ 43†	143 $\pm$ 9†
Female (8 weeks)								
Control (n=13)	330 $\pm$ 34	20 $\pm$ 1	1.4 $\pm$ 0.1	31 $\pm$ 1	13 $\pm$ 1	270 $\pm$ 50	78 $\pm$ 8	75 $\pm$ 3
Transgenic (n=4)	130 $\pm$ 23*	25 $\pm$ 1*	3.5 $\pm$ 0.7*	37 $\pm$ 3†	17 $\pm$ 1*	920 $\pm$ 310*	155 $\pm$ 47*	90 $\pm$ 1†
Female (19 weeks)								
Control (n=3)	255 $\pm$ 51	27 $\pm$ 2	4.5 $\pm$ 1.2	—	14 $\pm$ 1	510 $\pm$ 350	56 $\pm$ 8	59 $\pm$ 9
Transgenic (n=3)	74 $\pm$ 12†	52 $\pm$ 2*	28.1 $\pm$ 2.1*	—	29 $\pm$ 4†	5,690 $\pm$ 260*	155 $\pm$ 19*	138 $\pm$ 23†

Offspring of the UCP-DTA founder were weaned at age 2.7 weeks and housed separately in plastic cages at 24 °C with free access to food (Purina Formulab Chow 5008, 6.5% fat by weight). Transgenic mice and their control littermates were killed at the ages indicated. Food intake during the week before death is shown. Food was weighed at the beginning and end of each week and the differences were assumed to represent grams of food eaten per week. The cages were inspected carefully for spillage and none was noted. Total BAT UCP and total body lipid were determined as described in Fig. 2 legend. Serum glucose was assessed using an NADH enzyme-linked assay²² and serum insulin was determined using a rat insulin radioimmunoassay kit obtained from Linco Research, Inc. Serum cholesterol (procedure 339) and triglyceride (procedure 339) were quantified using kits obtained from Sigma. Because the triglyceride assay detects all serum glycerol (free and triglyceride derived), true serum triglyceride values were obtained by enzymatically assaying for free glycerol and adjusting the triglyceride value accordingly²². All values are shown as mean $\pm$ s.e.

* $P < 0.01$; † $P < 0.05$.

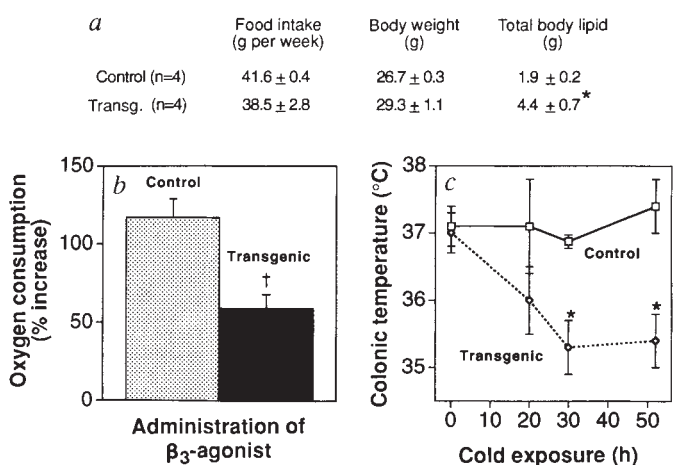
correlating with their relative deficiency of BAT. This degree of obesity at 16 days is comparable to that found in other murine models of genetic (*ob/ob*, *db/db*) or hypothalamic lesion (monosodium glutamate) induced obesity^{10,12}. As UCP-DTA mice age, they maintain their relative deficiency of BAT and obesity progresses (Table 1). By 19 weeks, UCP-DTA mice are extremely obese (>50% body fat). The two transgenic lines differ with respect to the status of BAT ablation over time. Whereas the UCP-DTA line has persistent BAT deficiency, the UCP-176 line has near complete regeneration of BAT by age 8 weeks (data not shown). Tissue regeneration following toxigen ablation has previously been observed and possible mechanisms have been discussed¹³. Regardless of its cause, whereas UCP-176 mice are extremely obese at 16 days, total body lipid stores have returned to normal by 8 weeks (data not shown). Regeneration of BAT followed by reversal of obesity provides compelling evidence that obesity in these mice is a consequence of BAT deficiency.

With advancing obesity, UCP-DTA mice develop several phenotypic abnormalities (Table 1); these include insulin resist-

ance as evidenced by hyperglycaemia and hyperinsulinaemia, hypertriglyceridaemia and hypercholesterolaemia. Corticosterone, which is elevated in nearly all genetic models of rodent obesity¹⁴, is normal in UCP-DTA mice (data not shown). Unexpectedly, UCP-DTA mice also develop hyperphagia, which begins at age 5–7 weeks and is uniformly observed in UCP-DTA mice over the age of 7 weeks. In the UCP-176 line, in which BAT regenerated, food intake was normal. As hyperphagia has so far been observed in only one line, it is possible that this phenotype is unrelated to ablation of BAT. One anatomical site where cellular ablation might cause hyperphagia is the hypothalamus¹⁴. To address this possibility, we have examined the brains of UCP-DTA mice histologically and found the appearance and volume of all hypothalamic nuclei to be normal. Additional evidence against a hypothalamic lesion is the fact the UCP-DTA mice are fertile (males and females) and grow normally (nasal anal length), processes that are sensitive to hypothalamic disturbance and impaired in other genetic models of rodent obesity¹⁴. Although provocative, the possibility of a

FIG. 2 Thermogenesis in heterozygous UCP-DTA mice. **a**, Total body lipid content of UCP-DTA mice before the development of hyperphagia. Male UCP-DTA mice and their control littermates were weaned at 2.7 weeks and group housed, two control and two transgenic mice per cage (four total per cage). At 5.5 weeks of age, they were separated and housed individually. Food intake was monitored over the following week after which the mice were killed and analysed for total body lipid content. **b**, Thermogenic response to CL 316,243 (CL), a β_3 -adrenoreceptor selective agonist. UCP-DTA (2 male and 8 female) and control (3 male and 4 female) littermates were weaned at age 2.7 weeks, then separated and housed individually at age 4.5 weeks. Mice were 62–69 days old at the time of CL administration. Male and female control mice responded similarly to CL. **c**, Colonic temperature during cold exposure. Female UCP-DTA ($n=6$) and control ($n=3$) littermates were weaned at age 2.7 weeks, individually housed at age 4.5 weeks (at 24 °C) and acutely exposed to cold to 4 °C at age 8 weeks. Colonic temperature was measured using a YSI model 43 telethermometer equipped with a series 500 probe (Yellow Springs Instrument Co., Inc., Ohio). All values are shown as mean $\pm$ s.e. * $P < 0.01$; † $P < 0.05$.

METHODS. Oxygen consumption was measured with computerized equipment which included a 1-litre chamber maintained at 28 °C, an air flow of 500 ml per min (regulated with a mass flowmeter, Brooks Instrument Division, Emerson Electric) and an oxygen analyser (Beckman Industrial Oxygen Analyzer model 755)²⁹. Mice were awake and unrestrained for the study. The resting rate of oxygen consumption was assessed when the mice were curled up and still, usually 1–2 h



after they had been placed in the chamber. CL was dissolved in water (10 mg ml⁻¹) and diluted in saline immediately before injection (1 μg per g, s.c.). In preliminary experiments with other mice, this dose exerted maximum effects on oxygen consumption. After injection of CL, mice were awake but still stretched out and panting and a constant rate of oxygen uptake was achieved after about 1 h.

TABLE 2 Summary of data obtained from 26-week-old non-transmitting UCP-DTA founders

	Sex	Status	Total BAT UCP (μ g per depot)	Body weight (g)	Total body lipid (g)	Food intake (g per week)
Controls ($n=4$)	Male	—	213 $\pm$ 83	39 $\pm$ 7	8 $\pm$ 2	31 $\pm$ 2
UCP-DTA-2	Male	Mosaic	107	60	20	39
UCP-DTA-3	Male	Infertile	83	65	26	39
Controls ($n=6$)	Female	—	ND	30 $\pm$ 4	7 $\pm$ 2	ND
UCP-DTA-4	Female	Mosaic	ND	46	17	ND

All animals were weaned at age 3 weeks and the founder mice were mated with nontransgenic animals. Four male and six female nontransgenic mice were housed individually and used as controls. The male controls were littermates of founders UCP-DTA-2 and -3 and the female controls were age-matched animals studied at another time. UCP-DTA-2 and UCP-DTA-4 were presumed to be mosaic because each failed to produce transgenic offspring and UCP-DTA-3 was infertile. All animals were housed individually at age 17 weeks and killed at age 26 weeks. Food intake was assessed during the week before death. Total body lipid was determined as described in Fig. 2 legend. For the control groups, values are shown as mean $\pm$ s.d. ND, not determined.

link between BAT deficiency and hyperphagia requires further study.

In addition to the above two lines, we have generated three non-transmitting UCP-DTA founders which were analysed at age 26 weeks (Table 2). These founders had diminished BAT as assessed by interscapular UCP content (not determined in UCP-DTA-4), were markedly overweight and had a large increase in total body lipid. Also, both UCP-DTA-2 and -3 were hyperphagic (not determined in UCP-DTA-4). The presence of brown fat deficiency, obesity and hyperphagia in these additional founder animals essentially rules out the possibility that these phenotypic features of the UCP-DTA line could be due to positional integration effects.

Because BAT is a site of thermogenesis, BAT deficiency might be expected to cause reduced energy expenditure, and experiments were performed to address this point (Fig. 2). When UCP-DTA mice were analysed before the development of hyperphagia (age 6 weeks), they were found to have increased total body lipid (Fig. 2a). The finding of increased energy stores in the absence of hyperphagia indicates that UCP-DTA mice have increased metabolic efficiency. Administration of β_3 -adrenoceptor agonists to rodents increases oxygen consumption by at least 100% (ref. 15). As β_3 -adrenoreceptors are found predominantly in brown and white fat¹⁶⁻¹⁸, it has been suggested that this response is due primarily to activation of BAT. However, when CL 316,243, an extremely selective β_3 -adrenoreceptor agonist¹⁹, was given to UCP-DTA mice, their thermogenic response was reduced by 50%, suggesting that these mice have a functional deficiency of BAT thermogenesis (Fig. 2b). Finally, when UCP-DTA mice were exposed to environmental cold, their core temperature gradually dropped by 1.7 °C, providing additional evidence of BAT dysfunction (Fig. 2c). The relatively small drop in body temperature is consistent with the partial BAT deficiency observed in UCP-DTA mice and with the view that brown fat mediates less than 40% of the thermogenic response to acute cold exposure²⁰, shivering being responsible for the remaining 60% (ref. 21).

The present study provides strong evidence that BAT plays a critical role in the regulation of energy balance in mice, and that BAT dysfunction can cause obesity. Also, the UCP-DTA mouse, by virtue of its normal fertility, linear growth and serum glucocorticoid levels, represents a valuable new model that may aid studies of the pathogenesis and treatment of human obesity and insulin resistance. □

- Cameron, D. P., Cutbush, L. & Opat, F. *Clin. exp. Pharmac. Physiol.* **5**, 41–51 (1978).
- Breitman, M. L. & Bernstein, A. in *Transgenic Animals* (eds Grosveld, F. & Kollias, G.) 127–145 (Academic, Boston, 1992).
- Bray, G. A. & York, D. A. *Physiol. Rev.* **59**, 719–809 (1979).
- Holloway, B. R. et al. *Br. J. Pharmac.* **104**, 97–104 (1991).
- Granneman, J. G., Lahners, K. N. & Chaudhry, A. *Molec. Pharmac.* **40**, 895–899 (1991).
- Muzzin, P. et al. *J. Biol. Chem.* **266**, 24053–24058 (1991).
- Nahmias, C. et al. *EMBO J.* **10**, 3721–3727 (1991).
- Bloom, J. D. et al. *J. Med. Chem.* **35**, 3081–3084 (1992).
- Foster, D. O. & Frydman, M. L. *Can. J. Physiol. Pharmac.* **57**, 257–270 (1979).
- Davis, T. R. A., Johnston, D. R., Bell, F. C. & Cremer, B. J. *Am. J. Physiol.* **198**, 471–475 (1960).
- Bergmeyer, H. U. *Methods in Enzymatic Analysis* 2nd edn (Academic, London, 1974).
- Boyer, B. B. & Kozak, L. P. *Molec. cell. Biol.* **11**, 4147–4156 (1991).
- Maxwell, I. H., Maxwell, F. & Glode, L. M. *Cancer Res.* **46**, 4660–4664 (1986).
- Maxwell, F., Maxwell, I. H. & Glode, L. M. *Molec. cell. Biol.* **7**, 1576–1579 (1987).
- Breitman, M. L., Rombola, H., Maxwell, I. H., Klintworth, G. K. & Berstein, A. *Molec. cell. Biol.* **10**, 474–479 (1990).
- Salmon, D. M. W. & Flatt, J. P. *Int. J. Obesity* **9**, 443–449 (1985).
- Desautels, M., Zaror-Behrens, G. & Himms-Hagen, J. *Can. J. Biochem.* **56**, 378–383 (1978).
- Ma, S. W. Y. & Foster, D. O. *Can. J. Physiol. Pharmac.* **64**, 1252–1258 (1986).

ACKNOWLEDGEMENTS. We thank A.-L. Kates for UCP western blotting reagents and methodology, I. Maxwell for pIB1 130-DT-A and pIB1-176, R. Lechan for hypothalamic histology, J. Heck-enlively for ophthalmological expertise, J.-P. Flatt for help with carcass analysis, T. Claus (American Cyanamid Co.) for CL 316,243, the National Research Council of Canada for the computerized equipment for measuring oxygen uptake, K. Herzberg of the Beth Israel Transgenic Facility for help with the generation of transgenic mice, and B. Spiegelman for critically reading our manuscript. This work was supported by grants from the NIH to B.B.L., J.S.F. and L.P.K., Miles, Inc. to B.B.L. and J.S.F. and the MRC of Canada to J.H.-H. This paper is dedicated to the memory of Patricia Usher.

A role for Fyn tyrosine kinase in the suckling behaviour of neonatal mice

Takeshi Yagi*†, Shinichi Aizawa*, Tomoyuki Tokunaga*, Yasuyo Shigetani*, Naoki Takeda* & Yoji Ikawa*‡§

* Laboratory of Molecular Oncology, Tsukuba Life Science Center, Institute of Physical and Chemical Research (RIKEN), Ibaraki 305, Japan

† Department of Biochemistry, Tokyo Medical and Dental University School of Medicine, Tokyo 113, Japan

NON-RECEPTOR-TYPE tyrosine kinases of the Src family, such as Src, Yes and Fyn, are strongly expressed in the brain and have been suggested to have an important function in the central nervous system¹⁻⁵. We generated Fyn-deficient mice by inserting the β -galactosidase gene (*lacZ*) into the *fyn* gene. The homozygous Fyn-mutant neonates from homozygous Fyn-deficient parents died because of a suckling problem. Neonates were, however, able to suckle milk normally when the homozygous mother's mammary glands had been activated by suckling of a heterozygous or wild-type pup. In these homozygous pups, the modified glomerular com-

Received 12 May; accepted 1 November 1993.

- Nicholls, D. G. & Locke, R. M. *Physiol. Rev.* **64**, 1–64 (1984).
- Klaus, S., Castella, L., Bouillaud, F. & Ricquier, D. *Int. J. Biochem.* **23**, 791–801 (1991).
- Rothwell, N. J. & Stock, M. J. *Nature* **281**, 31–35 (1979).
- Trayhurn, P. & Mercer, S. W. *Biochem. Soc. Trans.* **14**, 236–239 (1986).
- Himms-Hagen, J. *Prog. Lipid Res.* **28**, 67–115 (1989).
- Rothwell, N. J. & Stock, M. J. *Am. J. Physiol.* **257**, R253–R258 (1989).
- Palmiter, R. D. et al. *Cell* **50**, 435–443 (1987).
- Breitman, M. L. et al. *Science* **238**, 1563–1565 (1987).
- Jacobsson, A., Stadler, U., Grotzer, M. A. & Kozak, L. P. *J. Biol. Chem.* **260**, 16250–16254 (1985).
- Dubuc, P. U. *Metabolism* **25**, 1567–1574 (1976).
- Coleman, D. L. *Diabetologia* **14**, 141–148 (1978).

† Present address: Department of Neurobiology and Behavioral Genetics, National Institute for Physiological Sciences, Myodaiji, Okazaki 444, Japan.

§ To whom correspondence should be addressed.

FIG. 1 Lack of Fyn in Fyn-kinase-deficient mice. **a**, Targeting vector using the diphtheria toxin A fragment gene for negative selection^{6,15}. Exon 2 of *fyn* contains the translation initiation codon ATG. Black boxes represent exon 2 (ex.2) and exon 3 (ex.3). Probes used for Southern blot analyses are indicated by boxes. The construction of the targeting vector pHFZprNeoDT is described elsewhere⁷. **b**, Southern analysis of DNA isolated from pups between Fyn heterozygous mutants. Genomic DNA (10 µg) was digested with *EcoRI*. The probe was the indicated 0.8 kb *SphI*-*SspI* fragment of the third intron. Genotypes are shown on top. Plus and minus indicate the wild-type and *fyn*^{-/-} allele, respectively. **c**, Lysates from adult brains immunoprecipitated with rabbit anti-Fyn antibodies for *in vitro* kinase assays¹⁵. Adult brain extracts, 250 µg protein each, from wild-type (+/+), heterozygous (+/*fyn*⁻) and mutant (*fyn*^{-/-}/*fyn*⁻) littermates were immunoprecipitated with rabbit polyclonal anti-human Fyn antiserum¹⁶. Autophosphorylation kinase assay was done with 5 mCi [γ -³²P]ATP (300 Ci mmol⁻¹; Amersham), and separated in 8% SDS-polyacrylamide gels.

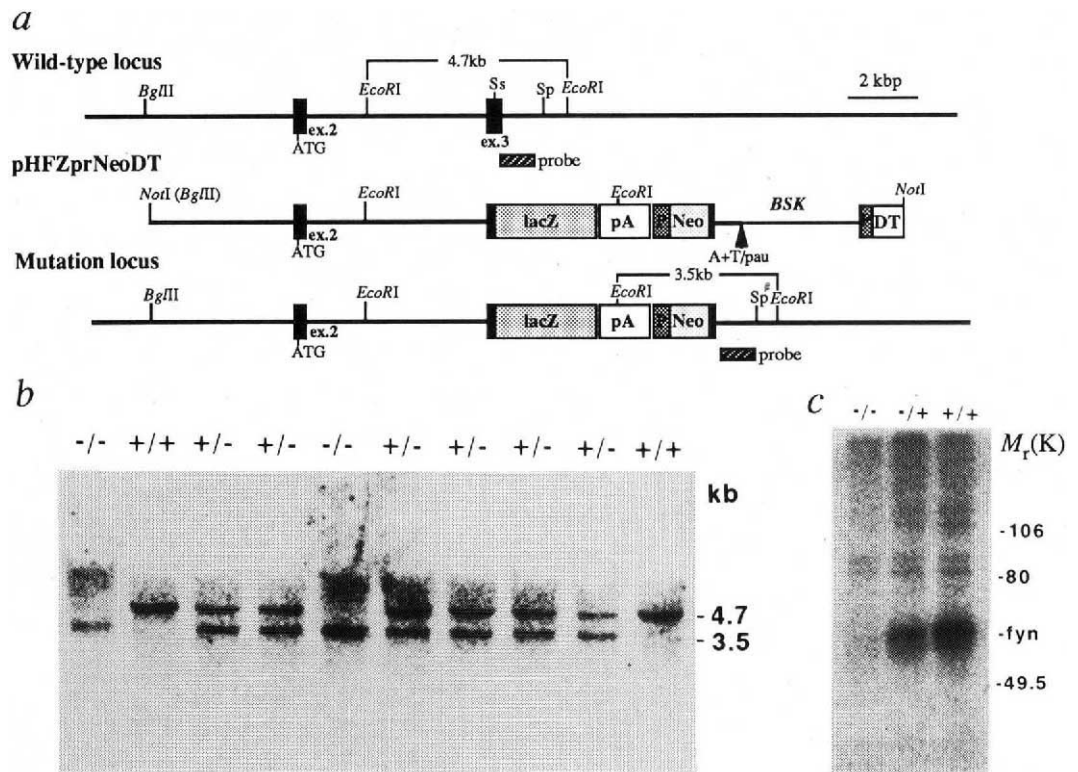


FIG. 2 **a**, **b**, **c** and **d**, Anatomical abnormalities of the MGC in 7-day-old Fyn-kinase-deficient pups. The MGC (asterisks) were stained with acetylcholine iodide (AChI) (**a** and **b**) or by Nissel's stain (**c** and **d**). The heterozygous or homozygous MGC are represented in **a** and **c** or **b** and **d**, respectively. Scale bars, 50 µm. **e**, Distribution of Fyn in the olfactory epithelium visualized by *lacZ* expression. The horizontal vibratome section (500 µm) of a heterozygous head on embryonic day 21 was stained by the X-gal reaction⁵. Arrowheads indicate nerves from the olfactory septal organ. NS; nasal septum. NC; nasal cavity. Scale bar, 500 µm. **f**, Anatomical abnormalities of the hippocampus of a 2-day-old homozygous pup. Frozen sections were stained with haematoxylin-eosin. The arrowhead indicates an undulating pyramidal cell layer in the CA3 region. DG; dentate gyrus. Scale bar, 500 µm. For AChI staining the excised olfactory bulb was fixed in PBS containing 4% paraformaldehyde. Serial frontal sections (10 µm) were treated with a solution of 80 mM Tris-malate buffer, pH 5.7, containing 10 mM sodium citrate, 3 mM cupric sulphate, 0.5 mM potassium ferricyanide and 0.05% AChI, for 60 min at room temperature.

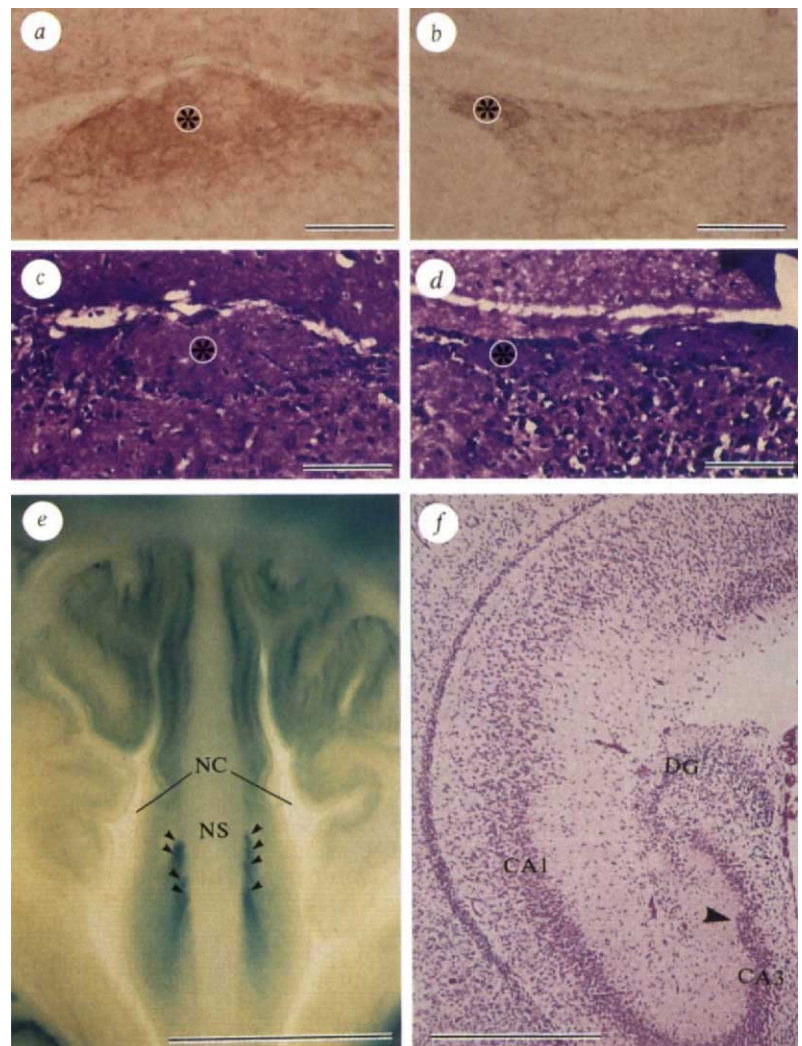


TABLE 1 Neonatal death of homozygous *Fyn*-deficient pups and their rescue experiment

(a) Neonatal death of homozygous pups from homozygous parents					
Father*	Mother*	No. of litters	No. of pups born	No. of dead pups	Neonatal death rate (%)
Het	WT	15	165	2	1.1
Het	Het	16	146	4	2.1
Hom	Hom	16	128	128	100
Hom†	Hom†	3	20	20	100
Hom	WT	13	129	6	4.6
WT	Hom	7	71	6	8.5
(b) Rescue of homozygous pups from neonatal death by addition of wild-type or heterozygous pups					
Genotype	Pups added‡	Number	Homozygous pups in a litter§ No. of dead pups (Death rate, %)		
WT		1	7	1	
WT		1	8	1	
WT		1	7	1	
WT		1	7	1	
WT		1	9	2	
WT		1	8	0	
WT		1	9	1	
WT (Total)		7	35	7 (20)	
Het		2	5	1	
Het		2	6	1	
Het		1	7	2	
Het		1	8	1	
Het		1	7	1	
Het (Total)		7	33	6 (18)	
Hom		2	6	8	
Hom		2	8	10	
Hom		1	7	8	
Hom		1	9	10	
Hom (Total)		6	30	36 (100)	
(c) Effect of nursing mothers on neonatal death					
Nursing mothers	Genotype	Pups foster-nursed			
		No. of pups examined	No. of dead pups (death rate %)		
WT¶	WT	98	5 (5.1)		
WT¶	Het	50	3 (6.0)		
WT¶	Hom	50	6 (12)		
Hom#	WT	50	8 (16)		
Hom#	Het	50	10 (20)		
Hom#	Hom	50	12 (24)		
Hom**	WT	50	9 (18)		
Hom**	Het	50	7 (14)		
Hom**	Hom	50	50 (100)		

* WT, wild-type; Het, heterozygous; Hom, homozygous.

† Results with the Fz62 strain of *Fyn*-deficient mice. All other experiments were done with the Fz42 strain.

‡ Wild-type, heterozygous or homozygous pups from other mothers were added on the morning of delivery.

§ Numbers of surviving homozygous pups were counted 15 days after birth.

|| On the morning of delivery, pups with the indicated genotype were transferred to nursing mothers (about 10 pups per mother) and the number of surviving pups was counted 15 days after birth.

¶ Wild-type mothers nursing wild-type pups obtained by mating with wild-type males.

Homozygous mothers nursing heterozygous pups obtained by mating with wild-type males.

** Homozygous mothers that delivered but did not nurse homozygous pups obtained by mating with homozygous males.

plex of the olfactory bulb, which had been suggested to play a role in perceiving pheromones, was abnormal in shape and reduced in size, and the hippocampal cell-layer was undulated. These results suggest that *Fyn* may be involved in the initial step of instinctive suckling behaviour in neonates.

Fyn-kinase-deficient mice were generated by replacing the SH2, SH3 and tyrosine kinase regions of *fyn* by *lacZ* in the cells of an embryonal stem (ES) cell line (TT2) derived from blastocysts of C57BL/6 × CBA F1 mice^{6,7}. We analysed two *fyn*-mutant strains from independent ES clones, Fz45 and Fz62.

Adult homozygous mutants were obtained by crosses between heterozygotes: 14 of 90 (16%) and 27 of 140 (19%) offspring were homozygous in the respective strains. In these homozygous mice, *Fyn* deficiency was confirmed by autophosphorylation kinase assay (Fig. 1c), and the presence of the unique region of *fyn* and *lacZ* fusion product by western blot analysis (data not shown).

Homozygous mutant mice appeared normal. But when they were intercrossed, all their pups withered and died within a few days of delivery (Table 1a). This neonatal death of homozygous pups from homozygous parents was observed in both the Fz45 and Fz62 strain. At autopsy, the external appearance of the pups and the architecture of their mouths were normal, but no milk was found in their stomachs. After delivery, these homozygous pups initially wriggled, crept about and squeaked normally, but did not attack the nipples, and no marks of suckling were apparent on the mother's nipples.

The neonatal death was first suspected to be due to a defect in lactation of the homozygous mothers. But no histological abnormality was found in their mammary glands. In addition, when homozygous females were mated with wild-type males, they nursed their pups normally (Table 1a). Thus the neonatal death was apparently not primarily due to an abnormality of the homozygous mothers.

The suckling problem in homozygous pups from homozygous parents but not from heterozygous or wild-type parents suggested that it might be overcome by heterozygous or wild-type littermates. To test this, one or two wild-type or heterozygous pups were added to litters of homozygous pups from homozygous parents immediately after delivery (Table 1b). As expected, it allowed homozygous mothers to nurse their homozygous pups normally. The addition of homozygous pups from other homozygous parents was not effective.

The above observation suggests that homozygous pups can suckle milk from lactating nipples but not from pre-lactating or unactivated nipples. To confirm this possibility, homozygous pups from homozygous parents were nursed by homozygous mothers that were nursing heterozygous pups obtained from crosses with wild-type males. As expected, homozygous pups could suckle milk from those activated mammary glands (Table 1c).

The processes leading to milk secretion are considered to be initiated by the pups by their contact with their mother, by their recognition of and attachment to the mother's nipples, and by biting the nipples⁸. After delivery, homozygous pups contacted and moved along the abdomen of their homozygous mother, but did not recognize or attach to her unactivated nipples. They could hold the mother's hair and could suckle activated nipples. Thus, the *Fyn*-deficient pups seem to have a defect in the second process. To examine this possibility, we placed 7-day-old homozygous pups, which had been saved by the addition of wild-type or heterozygous pups, by the side of the abdomen of anaesthetized, lactating or pre-lactating homozygous mothers. Thirty of 58 homozygous pups climbed up the abdomen and sucked from activated nipples within 10 min, but even within 15 min, only two of 58 found and sucked from unactivated homozygous nipples. These two positive cases may be due to spontaneous milk secretion or experience gained of suckling over the 7 days. It was thus concluded that *Fyn*-kinase-deficient pups had a defect in their suckling behaviour at the step of recognition and attachment to pre-lactating or unactivated nipples (this result is significant at less than 1% by comparison of the 99% confidence limits for the binomial distribution⁹). Among the control 7-day-old heterozygous pups, 16 of 28 found and suckled activated nipples and 10 of 28 found and suckled unactivated nipples within 10 min (suckling by heterozygous versus homozygous pups, $P < 0.001$ by Fisher exact probability test⁹).

Suckling is considered to be mediated by pheromones⁸. The stimuli are projected into a specific region, the modified glomerular complex (MGC) in the olfactory bulb^{10,11}, and finally pro-

cessed in the hippocampus. Fyn identified by *lacZ* expression has been detected as high levels in this pathway, including the olfactory bulb and hippocampal formation⁵. We tested for abnormality in this pathway by staining with acetylcholine iodide, a substrate of acetylcholinesterase (Fig. 2a)¹². The MGC, where the neural processes establish synaptic contacts, receives an input from the septal organ where suckling pheromones are perceived¹³. In heterozygous embryos, Fyn expression was found to be intense in the nerves of the septal organ just before birth (Fig. 2e), and as fine spots in the MGC (data not shown). Serial sections of the olfactory bulbs of Fyn-kinase-deficient pups revealed that the region of intense staining with acetylcholine iodide was abnormally small, and the region of weak staining surrounding it was distorted (Fig. 2b). The stained regions were oval in heterozygotes with a mean long axis of 200 μ m and short axis of 150 μ m ($n=4$), whereas these axes in homozygotes were 40 μ m and 30 μ m ($n=5$), respectively. These results suggest that in Fyn-kinase-deficient pups, the contacts of olfactory processed in the MGC were disordered, possibly resulting in defective suckling behaviour.

It is tempting to speculate that the structural abnormalities in the MGC and/or hippocampal formation (Fig. 2f, and its legend) alter sensitivities to pheromones. Possibly, Fyn-deficient mice cannot detect pheromones at the level secreted by unactivated nipples, but can detect them at the level secreted by activated nipples.

Finally, note that no suckling abnormality was detected in

Fyn-deficient mice by Grant *et al.*¹⁴. The reason for this discrepancy is unknown. They disrupted the gene in null, whereas in our mice the unique region of the *fyn* gene is expressed as a fusion product with β -galactosidase, possibly occupying the original space for Fyn. $\square$

Received 2 August; accepted 4 October 1993.

1. Sugrue, M. M. *et al.* *J. Neurosci.* **10**, 2513–2527 (1990).
2. Zhao, H. *et al.* *Oncogene* **6**, 1725–1733 (1991).
3. Cartwright, A. C. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **85**, 3348–3352 (1988).
4. Umemori, H. *et al.* *Molec. Brain Res.* **16**, 303–310 (1992).
5. Yagi, T. *et al.* *Oncogene* (in the press).
6. Yagi, T. *et al.* *Analyt. Biochem.* **214**, 77–86 (1993).
7. Yagi, T. *et al.* *Analyt. Biochem.* **214**, 68–76 (1993).
8. Blass, E. M. & Teicher, M. H. *Science* **210**, 15–22 (1980).
9. Diem, K. & Lentner, C. *Wissenschaftliche Tabellen* 7th edn (Geigy Pharmazeutika, Wehr, Baden, 1968).
10. Teicher, M. H. *et al.* *Brain Res.* **194**, 530–535 (1980).
11. Greer, C. A. *et al.* *J. Neurosci.* **12**, 1744–1759 (1992).
12. Ojima, H. *et al.* *Anat. Embryol.* **178**, 481–488 (1988).
13. Pedersen, P. E. & Benson, T. E. *J. comp. Neurol.* **252**, 555–562 (1986).
14. Grant, S. G. N. *et al.* *Science* **258**, 1903–1910 (1992).
15. Yagi, T. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **87**, 9918–9922 (1990).
16. Katagiri, T. *et al.* *Molec. cell. Biol.* **9**, 4914–4922 (1989).

ACKNOWLEDGEMENTS. We thank E. A. Ichihara and H. Niki for help in the preparation of the manuscript, T. Yamamoto, University of Tokyo, for anti-Fyn antibody, H. Ojima for advice on the AChI staining method, T. Tomooka for histological examination of mammary glands and H. Tomikawa and Y. Tomita for maintenance of mice. This study was partly supported by a research contract from the Research Coordination Bureau, Science and Technology Agency (STA) of the Japanese Government, and by a Grant-in-aid for Cancer Research from the Ministry of Education, Science and Culture of the Japanese Government. T.Y. is a recipient of an STA Fellowship (Special Researcher's Basic Science Program).

Gephyrin antisense oligonucleotides prevent glycine receptor clustering in spinal neurons

J. Kirsch, I. Wolters, A. Triller* & H. Betz†

Department of Neurochemistry, Max-Planck-Institute for Brain Research, Deutschordenstrasse 46, 60528 Frankfurt/Main, Germany
* Neurobiologie Cellulaire, Inserm U261, Département des Biotechnologies, Institut Pasteur, 28 rue du Dr Roux, 750 Paris Cedex 15, France

EACH neuron in the mammalian brain carries many postsynaptic membrane specializations containing high densities of receptors that mediate signal transduction upon neurotransmitter release from the apposed nerve terminal¹. Little is known about the mechanisms by which receptors are transported to and anchored at postsynaptic sites, but extracellular² as well as intracellular³ components may be involved. Ultrastructural studies have shown that the peripheral membrane protein gephyrin⁴, which co-purifies with the postsynaptic inhibitory glycine receptor (GlyR) upon affinity chromatography^{5,6}, is situated on the cytoplasmic face of glycinergic postsynaptic membranes^{7–9}. Moreover, gephyrin binds with high affinity to polymerized tubulin and has been postulated to link the GlyR to the subsynaptic cytoskeleton¹⁰. Here we report that treatment of rat spinal neurons in culture with gephyrin antisense oligonucleotides prevents the formation of GlyR clusters in the dendritic plasma membrane. Thus, gephyrin is essential for localizing the GlyR to presumptive postsynaptic plasma membrane specializations.

Glycinergic synapses are abundant in spinal cord, and the ontogenetic expression of the GlyR is conserved in primary

cultures prepared from rodent spinal cord at embryonic day 14 (ref. 11). After 6–10 days *in vitro*, more than 90% of the neurons in these cultures express functional GlyRs, as revealed by electrophysiology¹² and immunostaining¹³. GlyR antigen was detected by the α -subunit-specific monoclonal antibody 4a (ref. 14) as early as day 3 *in vitro*, before the formation of synaptic contacts¹³. Confocal laser scanning microscopy showed that at this stage the GlyR is mainly localized intracellularly (Fig. 1a). On about day 6 *in vitro*, the number of cells expressing GlyR increased (Fig. 1c). Optical sectioning with the confocal microscope showed that most of the fluorescent signal was localized intracellularly; only a few small GlyR aggregates were seen at the surface of the proximal part of the neurites (Fig. 1c). Their number and size increased further upon differentiation, and at around day 10 *in vitro*, many small clusters of GlyR immunoreactivity were visible on the cell body and along the neuritic surface, in addition to some residual intracellular antigen (Fig. 1e). Previous ultrastructural studies on adult spinal cord have shown that these GlyR clusters correspond to postsynaptic membrane specializations^{7–9}. Double labelling with an antiserum directed against synaptic vesicle proteins indicates that most of these GlyR clusters are indeed apposed to presynaptic terminals (J.K., in preparation).

The distribution of gephyrin as revealed by antibody 7a (ref. 14) was very different from that of the GlyR. At day 3 *in vitro*, gephyrin immunoreactivity was mainly localized at sites of focal cell attachment, in addition to a diffuse cytoplasmic staining (Fig. 1b). As morphological differentiation continued, these gephyrin-rich domains increased in number and size, and around day 6 *in vitro* they were prominent in the cell periphery and along neurites (Fig. 1d). Gephyrin immunoreactivity in the cytoplasm gradually decreased until day 10. At this stage punctate aggregates outlined the contours of many neuronal somata and neurites (Fig. 1f). Double-labelling experiments confirmed that at day 10, GlyR immunoreactivity co-localized with the gephyrin aggregates (Fig. 2). These results suggest that gephyrin associates with the GlyR after its appearance at the cell surface. Gephyrin aggregates are detected earlier than GlyR clusters.

† To whom correspondence should be addressed.

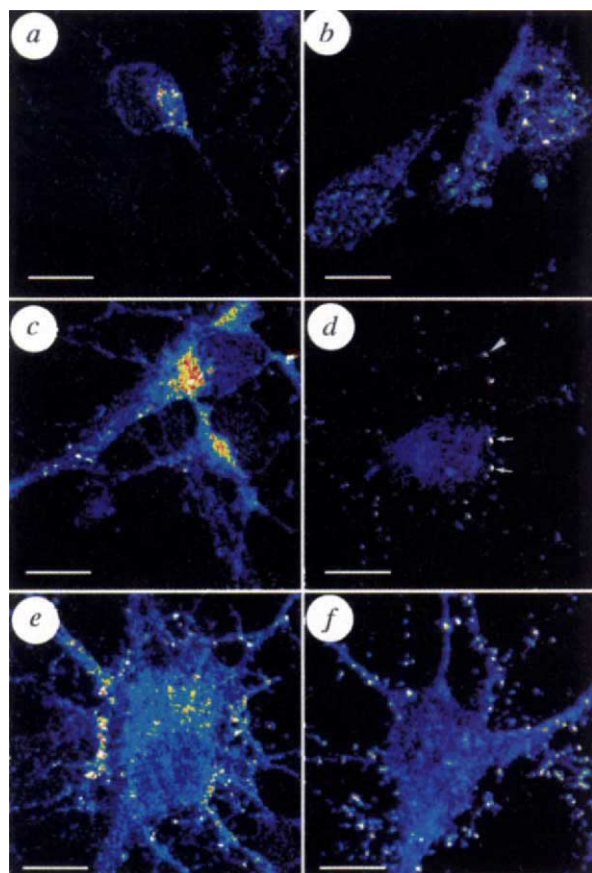


FIG. 1 Look-through projections of confocal sections of primary neurons from embryonic rat spinal cord stained with the GlyR-specific monoclonal antibody (mAb) 4a (left panels) and the gephyrin-specific mAb 7a (right panels). *a* and *b*, Day 3 *in vitro*. GlyR immunoreactivity is detected intracellularly, whereas gephyrin is localized at focal adhesion plaques. *c* and *d*, Day 6 *in vitro*. GlyR aggregates become visible on the proximal neurites (arrowheads); gephyrin is localized close to the membrane in the periphery of the cell soma (arrows) and along the neurites (arrowheads). *e* and *f*, At day 10 *in vitro*, many GlyR clusters are seen on the cell body and along the neuritic extensions. Scale bars, 10 μ m. METHODS. The spinal cord of E14 rat embryos (Wistar) was removed and mechanically dissociated by trituration. Cells were plated onto glass coverslips (8 mm diameter) coated with poly-DL-ornithine (Sigma) and fetal calf serum (Gibco) at a density of 120,000 per well. Growth in a chemically defined medium as described¹³ resulted in an initially 95% pure neuronal culture as judged by immunostaining with a monoclonal antibody directed against microtubule-associated protein 2 (ref. 29). At the times indicated, coverslips were processed for mAb4a and mAb7a (ref. 14) immunofluorescence as described²⁷. Antigen-antibody complexes were visualized using fluorescein isothiocyanate or carbocymethylindocyanin-3-coupled goat anti-mouse IgG F(ab')₂ (Dianova, Hamburg). Optical sections of 0.1 μ m pixel size were acquired with a Sarastro 2000 confocal laser scanning microscope. Projections were calculated from these sections with ImageSpace software (Molecular Dynamics).

Thus, the assembly of gephyrin-rich domains at the plasma membrane may precede the formation of glycinergic membrane specializations.

To analyse whether membrane-associated gephyrin is required for the formation of GlyR clusters, spinal cord cultures were treated with gephyrin antisense oligonucleotides. As determined by immunofluorescence microscopy and dot immunoassay with antibody 7a (Fig. 3a), gephyrin expression at days 5 and 7 *in vitro* was efficiently reduced by the daily addition to the culture medium of an antisense phosphorothioate oligonucleotide covering the translation initiation codon (nucleotide positions 261–285) of the gephyrin complementary DNA sequence⁴. The corresponding sense oligonucleotide had no effect on gephyrin expression. Neither oligonucleotide affected cell morphology, nor did

they interfere with expression of GlyR protein as detected by quantitative analysis of antibody 4a binding (Fig. 3b).

Confocal microscopy revealed GlyR immunoreactivity in cultures treated with antisense oligonucleotide; the intracellular distribution was similar to that seen in control cultures or cultures treated with sense oligonucleotides at day 3 *in vitro* (results not shown). However, the formation of GlyR clusters, which occurred in sense-oligonucleotide-treated cultures around day 6, was not observed in cells treated with the antisense oligonucleotide (Fig. 4a, b). Only 0.3% of the cells showed punctate immunostaining with antibody 4a, as opposed to >60% in control or sense-oligonucleotide-treated cultures (Fig. 4c). Moreover, the number of GlyR clusters per cell dropped from about 65 in control and sense-oligonucleotide-treated cultures to 1.5 after antisense-oligonucleotide treatment (Fig. 4d). The percentage of neurons reactive with antibody 4a, however, was very similar under all culture conditions tested (see Fig. 4 legend). A similar but less pronounced inhibition of GlyR cluster formation was also observed upon treatment with another antisense phosphorothioate oligonucleotide complementary to nucleotide positions 182–203 of the gephyrin cDNA (data not shown). The corresponding sense oligonucleotide and an unrelated phosphorothioate oligonucleotide of similar composition had no effect.

FIG. 2 Spinal cord neuron (day 10 *in vitro*) double-labelled with mAb 4a (green) and mAb 7a (red). At the cell periphery, GlyR and gephyrin immunoreactivities are co-localized in foci (yellow). A few foci are single-labelled by either mAb 7a (double arrowhead) or mAb 4a (arrowheads). Analysis of the individual sections showed that foci single-labelled by mAb 4a were exclusively found within the cell body, whereas all mAb 7a-positive foci were at the cell surface. The projection was reconstructed from confocal sections. Scale bar, 10 μ m.

METHODS. Spinal cord cultures were prepared as described in the legend to Fig. 1. At day 10 *in vitro*, cells were fixed as already described and processed for simultaneous immunodetection of gephyrin and GlyR with mAbs 4a and 7a as in ref. 9. Appropriate controls in the absence of primary antibodies confirmed the specificity of the immunolabelling.

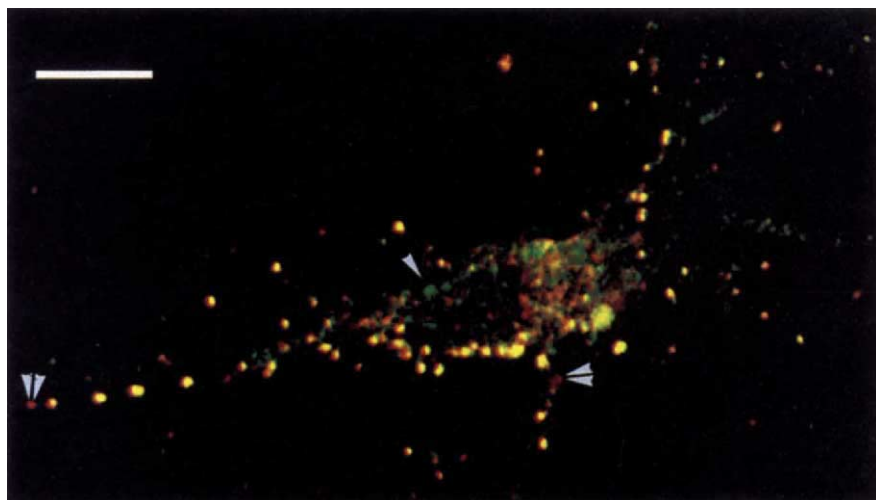


FIG. 3 Effect of antisense oligonucleotide treatment on gephyrin and GlyR protein levels in spinal cord cultures. Control cultures, ■; sense-treated, □; antisense-treated, ▨. *a*, Gephyrin immunoreactivity decreased to background after 5 days of treatment with antisense oligonucleotide, whereas gephyrin expression in cultures treated with sense oligonucleotide was not significantly affected. *b*, GlyR expression, as monitored by mAb 4a immunoreactivity, increased continuously during antisense oligonucleotide treatment. Note that GlyR expression in sense- and antisense-oligonucleotide-treated cultures was not significantly different from that in control cultures. Data represent the mean $\pm$ s.d. of triplicate determinations.

METHODS. Primary spinal neurons were plated at a density of 240,000 cells per well on glass coverslips as described in Fig. 1 legend. Phosphorothioate-modified oligonucleotides covering the translation start codon (nucleotide positions 261–285) of the gephyrin cDNA sequence⁴ in sense and antisense orientation were custom-synthesized by Appligene (Heidelberg). Starting the day after plating, the HPLC-purified oligonucleotides were applied every 24 h in 2 μ l medium to a final concentration of 5 μ M. Control cultures received 2 μ l of normal medium every 24 h. Cells were dislodged and solubilized in 100 μ l 50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 4% (w/v) sodium deoxycholate, 40% (v/v) methanol and protease inhibitors³⁰. Samples were spotted onto nitrocellulose membranes (Schleicher & Schuell), and their GlyR and gephyrin contents determined by quantitative immunoassay³⁰ using alkaline-phosphatase-conjugated anti-mouse IgG (Promega) as secondary antibody and *p*-nitrophenyl-phosphate (Sigma) as chromogenic substrate.

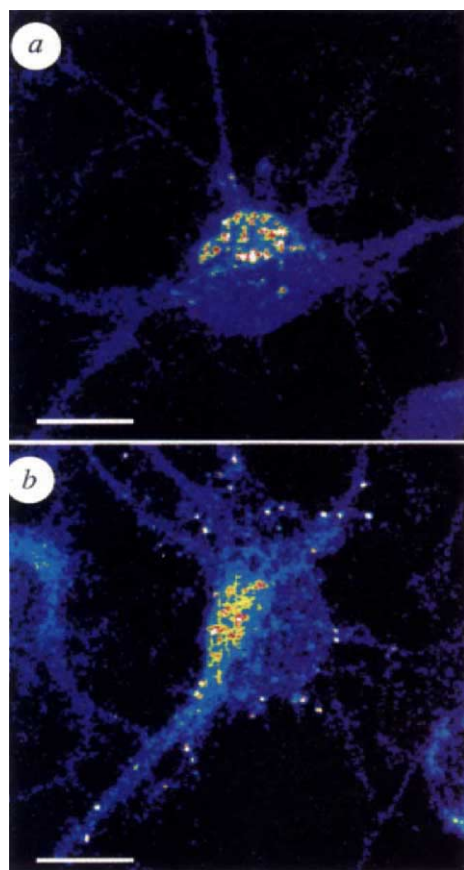
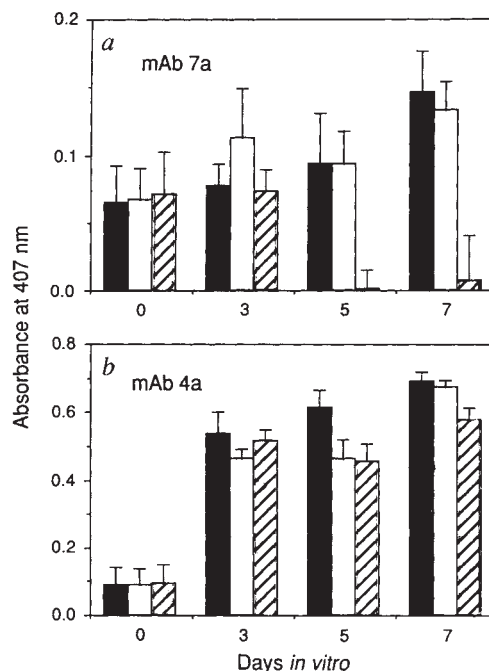
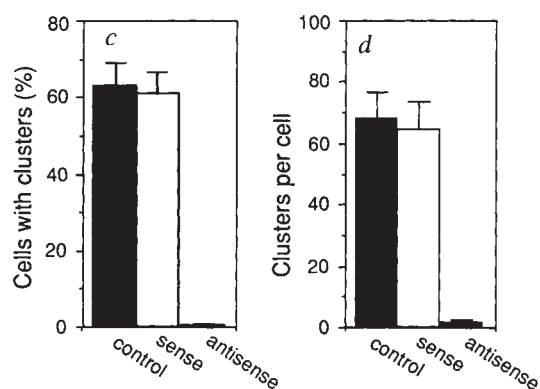


FIG. 4 GlyR immunoreactivity in oligonucleotide-treated spinal neurons stained with mAb 4a. *a* and *b*, Images reconstructed from confocal sections. *a*, Cultures treated with gephyrin antisense oligonucleotide lack peripheral GlyR clusters, although GlyR antigen levels were not affected by this treatment (Fig. 3). Analysis of confocal sections showed that this GlyR immunoreactivity was confined to vesicular structures within the cell body and absent from the cell surface. *b*, Control cultures exposed to the corresponding sense oligonucleotide display a distribu-



tion of GlyR immunoreactivity indistinguishable from that of untreated cultures grown in parallel (Fig. 1 *a, c*). Scale bars, 10 μ m. *c* and *d*, Quantitative evaluation of oligonucleotide effects. *c*, At day 6 *in vitro*, $63.3 \pm 6.3\%$ ($n=550$) of the cultivated cells are stained with mAb 4a. All of these show GlyR clusters at this stage. Similar results ($60.7 \pm 5.8\%$ mAb 4a-immunoreactive cells, $n=619$) were obtained in cultures treated with sense oligonucleotides. The percentage of cells with GlyR clusters is dramatically reduced in cultures treated with antisense oligonucleotides ($0.3 \pm 0.4\%$, $n=544$), although the percentage of immunoreactive cells was not significantly different ($59.6 \pm 5.7\%$, $n=544$). *d*, In control cultures, the mean number of clusters per cell was 68.2 ± 8.7 ($n=10$). No significant difference was observed in cultures treated with sense oligonucleotide (64.4 ± 8.8 , $n=10$). Treatment with gephyrin antisense oligonucleotide decreased the mean number of clusters per cell to 1.5 ± 0.7 ($n=12$).

METHODS. Spinal cord cultures were prepared as described in Fig. 1 legend. Oligonucleotides were applied daily from day 1 to day 6 *in vitro* (see Fig. 3 legend). Cultures were processed for immunostaining with mAb 4a and analysed by confocal microscopy (Fig. 1 legend). To quantify oligonucleotide effects at the cellular level, five visual fields per culture were chosen randomly, and the number of cells displaying GlyR immunoreactivity and/or clusters were counted. Similarly, the number of clusters per cell was determined from individual cells. For the determination of clusters per cell in antisense-treated cultures, only cells with clusters were considered; these data therefore represent an overestimation of the mean. All data shown represent means $\pm$ s.d. of three independent experiments.

The effects of antisense oligonucleotide inhibition were fully reversible. Oligonucleotide withdrawal at day 6 *in vitro* resulted in the recovery of gephyrin expression and the formation of GlyR clusters after another five days *in vitro*. Neither the percentage of cells with GlyR clusters ($39.6 \pm 0.9\%$; $n = 521$) nor the number of clusters per cell (73.8 ± 5.8 , $n = 9$) were significantly different from the values found in a parallel control ($44.1 \pm 3.7\%$, $n = 508$; and 82.7 ± 13.1 , $n = 12$) or in sense-oligonucleotide-treated ($42.4 \pm 2.4\%$, $n = 518$; and 72.0 ± 4.9 , $n = 10$) culture dishes.

These results strongly support the view that gephyrin is required for the formation of GlyR-rich domains in the dendritic plasma membrane. A similar function has been proposed for the peripheral protein of Mr 43,000 (43K) that co-distributes with postsynaptic nicotinic acetylcholine receptors (nAChRs) at vertebrate neuromuscular junctions and the electromotor synapse of *Torpedo* electric organ¹⁵⁻¹⁷. Heterologous expression of the cloned 43K protein in *Xenopus* oocytes causes clustering of co-expressed nAChRs in the oocyte plasma membrane¹⁸. Moreover, transfection of fibroblast cell lines expressing recombinant nAChRs with cDNA encoding the 43K protein causes reorganization of the receptors into discrete 43K protein-rich plasma membrane domains¹⁹. There is evidence that actin can bind to the 43K protein *in vitro*²⁰. But although immunofluorescence studies have demonstrated the presence of actin at receptor-rich domains of cultured rat myotubes²¹, actin bundles do not co-localize with nAChRs at the electromotor synapse of *Torpedo*²².

The molecular mechanisms by which gephyrin mediates GlyR clustering can at present only be a subject of speculation. The high-affinity binding of gephyrin to tubulin may be important in this process. The occurrence of 'membrane-bound' tubulin in brain synaptosomes has frequently been reported, although there is some controversy as to whether tubulin is indeed a major postsynaptic component²³. Also, there is a subsynaptic array of cold-stable acetylated tubulin at the neuromuscular junction²⁴, and tubulin consistently copurifies with postsynaptic densities isolated from mammalian brain²⁵. Thus, submembranous microtubular domains may be a general feature of postsynaptic specializations²⁶ and provide a scaffold for anchoring receptors under nerve terminals. Recent immunohistochemical²⁷ and *in situ* hybridization²⁸ studies have indicated a widespread expression of gephyrin in the rat central nervous system. Gephyrin may therefore also be involved in regulating the cell-surface distribution of other ligand-gated ion channel proteins (for discussion, see refs 4, 28). Co-expression of gephyrin deletion mutants with GlyR subunits should help to identify the domains of this polypeptide that interact with receptors and cytoskeletal elements. □

23. Stephens, R. E. *Biol. Cell* **57**, 95-110 (1986).
24. Jasmin, B. J., Changeux, J.-P. & Cartaud, J. *Nature* **344**, 673-675 (1991).
25. Blomberg, F., Cohen, R. S. & Siekevitz, P. *J. Cell Biol.* **74**, 204-225 (1977).
26. Hirokawa, N. & Heuser, J. E. *J. Neurocytol.* **11**, 487-510 (1982).
27. Kirsch, J. & Betz, H. *Brain Res.* **621**, 301-310 (1993).
28. Kirsch, J., Malosio, M.-L., Wolters, I. & Betz, H. *Eur. J. Neurosci.* **5**, 1109-1117 (1993).
29. Kirsch, J., Zutra, A. & Littauer, U. Z. *J. Neurochem.* **55**, 1031-1041 (1990).
30. Becker, C.-M., Hoch, W. & Betz, H. *J. Neurochem.* **53**, 124-131 (1989).

ACKNOWLEDGEMENTS. We thank V. O'Connor for his comments. This work was supported by the Deutsche Forschungsgemeinschaft (Schwerpunktprogramm Funktionelle Domänen und Leibniz-Programm), Association Française contre les Myopathies, and Fonds der Chemischen Industrie.

Potential virulence determinants in terminal regions of variola smallpox virus genome

Robert F. Massung*, Joseph J. Esposito*†, Li-ing Liu‡§, Jin Qi*, Theresa R. Utterback‡§, Janice C. Knight*, Lisa Aubin‡§, Thomas E. Yuran*, Joseph M. Parsons*, Vladimir N. Loparev*, Nickolay A. Selivanov‡, Kathleen F. Cavallaro*, Anthony R. Kerlavage‡§, Brian W. J. Mahy* & J. Craig Venter‡§

* Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia 30333, USA

‡ National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20892, USA

§ The Institute for Genomic Research, Gaithersburg, Maryland 20878, USA

SMALLPOX eradication culminated the most successful antimicrobial campaign in medical history¹. To characterize further the linear double-stranded DNA genome of the aetiological agent of smallpox, we have determined the entire nucleotide sequence of the highly virulent variola major virus, strain Bangladesh-1975 (VAR-BSH; 186,102 base pairs, 33.7% G + C; Genbank accession number, L22579). Here we highlight features of the molecule and focus on a few of the 187 putative proteins that probably contribute to pathogenicity and virus host-range properties. One hundred and fifty proteins were markedly similar to those of vaccinia virus (smallpox vaccine), for which a complete sequence has been reported^{2,3} for strain Copenhagen (VAC-CPN; 191,636 base pairs, 33.3% G + C). The remaining 37 proteins reflected variola-specific sequences or open reading frame divergences for variant proteins, which are often truncated or elongated compared with their vaccinia counterparts.

Smallpox was a strictly human disease, marked by respiratory or skin infection with the orthopoxvirus variola virus, followed by virus growth in lymphoid organs, viraemia, pustular rash, and fever with toxæmia and complications that led to case fatality rates ranging up to 40%. In contrast, vaccinia virus vaccination produces a self-limiting, localized infection, rarely with side-effects^{1,4}. Thus, it was of interest to compare the DNA sequences of VAR-BSH and VAC-CPN.

Orthopoxvirus DNA restriction mapping and gene product studies, mainly with vaccinia virus, have shown a large, conserved central DNA region, predominantly containing genes essential for virus structure and replication, flanked by hypervariable terminals containing genes contributing to phenotypic variability, arrays of near-terminal repeats, and covalently closed hairpin ends⁴⁻⁸. The close relationship of VAR-BSH and VAC-

Received 8 July; accepted 25 October 1993.

1. Peters, A., Palay, S. L. & Webster, H. de F. *The Fine Structure of the Nervous System* (Oxford Univ. Press, Oxford, 1992).
2. McMahan, U. J. & Wallace, B. G. *Dev. Neurosci.* **11**, 227-247 (1989).
3. Froehner, S. C. A. *Rev. Neurosci.* **16**, 347-368 (1993).
4. Prior, P. et al. *Neuron* **8**, 1161-1170 (1992).
5. Pfeiffer, F., Graham, D. & Betz, H. *J. Biol. Chem.* **257**, 9389-9393 (1982).
6. Schmitt, B., Knaus, P., Becker, C.-M. & Betz, H. *Biochemistry* **26**, 805-811 (1987).
7. Triller, A., Cluzeaud, F., Pfeiffer, F., Betz, H. & Korn, H. *J. Cell Biol.* **101**, 683-688 (1985).
8. Altschuler, R. A., Betz, H., Parakkal, M., Reeks, K. & Wenthold, R. *Brain Res.* **369**, 316-320 (1986).
9. Triller, A., Cluzeaud, F. & Korn, H. *J. Cell Biol.* **104**, 947-956 (1987).
10. Kirsch, J. et al. *J. Biol. Chem.* **266**, 22242-22245 (1991).
11. Hoch, W., Betz, H. & Becker, C.-M. *Neuron* **3**, 339-348 (1990).
12. Bormann, J., Hamill, O. P. & Sakmann, B. *J. Physiol., Lond.* **385**, 243-286 (1987).
13. Nicola, M.-A., Becker, C.-M. & Triller, A. *Neurosci. Lett.* **138**, 173-178 (1992).
14. Pfeiffer, F., Simler, R., Grenningloh, G. & Betz, H. *Proc. natn. Acad. Sci. U.S.A.* **81**, 7224-7227 (1984).
15. Sealock, R., Wray, B. & Froehner, S. C. *J. Cell Biol.* **98**, 2239-2244 (1984).
16. Bridgman, P. C., Carr, C., Pedersen, S. E. & Cohen, J. B. *J. Cell Biol.* **105**, 1829-1846 (1989).
17. Nghiem, H. O. et al. *Proc. natn. Acad. Sci. U.S.A.* **80**, 6403-6407 (1983).
18. Froehner, S. C., Luetje, C. W., Scotland, P. B. & Patrick, J. *Neuron* **5**, 403-410 (1990).
19. Phillips, W. D. et al. *Science* **251**, 568-570 (1991).
20. Walker, J. H., Boustead, C. M. & Witzemann, V. *EMBO J.* **3**, 2287-2296 (1984).
21. Bloch, R. J. *J. Cell Biol.* **102**, 1447-1458 (1986).
22. LaRoche, W. J., Witzemann, V., Fiedler, W. & Froehner, S. C. *J. Neurosci.* **10**, 3460-3467 (1990).

† To whom correspondence should be addressed.

TABLE 1 Genome location and number of predicted proteins of VAR-BSH and homology with correlates in VAC-CPN or VAC-WR

VAR-BSH <i>Hind</i> III fragment*	Number predicted proteins§	Number homologous to vaccinia proteins†			Number truncated‡	
		≥90%	30–90%	Variola-specific	VAR-BSH	vaccinia
D	18	12	5	1	8	3
P	2	2	—	—	—	—
O	3	3	—	—	2	—
C	21	15	6	—	3	—
E	11	10	1	—	—	—
Q	2	2	—	—	—	—
K	8	8	—	—	—	—
H	9	9	—	—	—	—
M	5	5	—	—	—	—
L	6	6	—	—	—	—
I	7	7	—	—	—	—
F	10	10	—	—	—	—
N	3	3	—	—	—	—
A	49	40	5	4	6	1
J	8	6	2	—	1	—
B	22	11	4	7	4	1
G	3	1	2	—	—	2
Totals:	187	150	25	12	24	7

* *Hind*III fragments listed as arranged in VAR-BSH map (Fig. 1).

§ Predicted proteins of ≥65 amino-acids initiating at first methionine ATG.

† VAR-BSH proteins compared with predicted proteins of VAC-CPN, or if absent or disrupted, then with correlate in VAC-WR; per cent identity of <30% by FASTA amino-acid pairings²³ were considered virus-specific.

‡ Truncated means that >10% of amino-acid residues are lost.

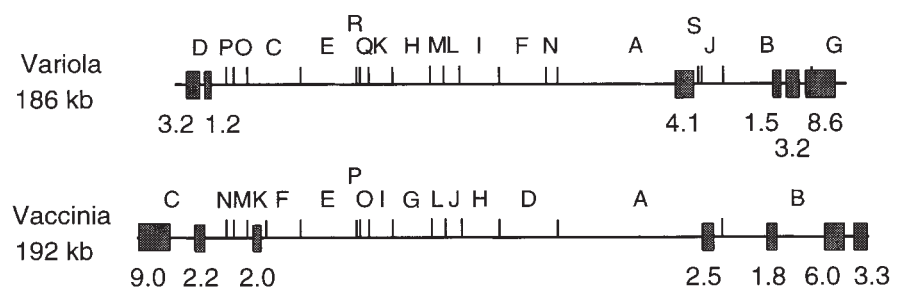
CPN genomes is seen by aligning their DNA *Hind*III maps, as shown in Fig. 1; but as depicted, alignment of the sequences reveals six variola- and seven vaccinia-specific terminal DNA segments of ≥1 kilobase pair (kb). At each end, VAR-BSH showed (A+T)-rich telomeres similar to vaccinia virus^{2,4}, followed by one left- and two right-end small clusters of directly repeated 69-base-pair (bp) elements. The asymmetrical small arrays distinguish variola DNA from DNA of many other orthopoxviruses, which have rather large, mirror-imaged arrays containing multiple, variable-sized repeat clusters near the ends of large inverted terminal repeat (ITR) regions^{4,7}.

VAR-BSH has 81 plus-strand and 106 minus-strand closely spaced major open reading frames (ORFs) for proteins of ≥65 amino acids; 50 minor ORFs (≥65 residues) could potentially initiate within major ORFs, but no messenger RNA splicing and virtually no internal initiation occur with poxviruses⁶. Transcripts emanate bidirectionally from central region DNA and predominantly from the plus strand of the left terminal region and the minus strand of the right terminal region. Central

DNA-encoded proteins contained amino-acid sequences well conserved compared with those of vaccinia; but proteins from VAR-BSH terminal region ORFs, compared with vaccinia and other sequenced orthopoxvirus homologues, reflected single or multiple frameshifts or larger insertions and deletions that often translated to elongated or truncated variants. Deletions often reflected small direct repeats within the larger homologue, analogous to the ORF fragmentation described for variola strain Harvey compared with counterparts in vaccinia, and between ORFs of VAC-CPN and vaccinia strain Western Reserve (VAC-WR)^{2,3,9,10}.

To illustrate the similarities and differences between VAR-BSH and VAC-CPN predicted proteins, Table 1 shows the number of proteins initiating within individual VAR-BSH *Hind*III fragments and the number of proteins with high, moderate or low homology to vaccinia counterparts. Eleven of twelve variola-specific ORFs encoded small proteins (<130 residues) largely derived from frameshifts fragmenting sequences well conserved between VAC-CPN and VAR-BSH. VAR-BSH encoded 24

FIG. 1 Nucleotide homology of variola Bangladesh and vaccinia Copenhagen DNA molecules. *Hind*III restriction maps that depict virus-specific regions determined by pairing the two virus DNAs to establish non-homologous segments of ≥1 kb (shaded; sizes in kb). Briefly, our sequencing strategy, which is fully detailed elsewhere (L.L. et al., in preparation), used double-strand templates derived by sub-cloning from a master set of plasmid-cloned *Hind*III, *Sac*I and *Bst*EII fragments¹⁷ (included all VAR-BSH DNA but the hairpin-like ends) either in an exonuclease III-generated, nested deletion protocol²⁴ (80% of the genome), or, when suitable restriction sites were lacking, in a DNase I-digested, random shotgun protocol²⁵ (20% of the genome). For sequencing of the plasmid templates, fluorescent dye-labelled terminator dideoxynucleotides were used in temperature-cycled, *Taq* polymerase-based, Sanger-type reactions²⁶ robotically performed with an Applied Biosystems Catalyst System, or done manually with a Perkin-Elmer Cetus Model 9600 thermocycler. Reaction products were analysed using the ABI Model 373A Sequencing System as described²⁷, and output sequences were



assembled into contigs using the ABI INHERIT Assembler. Direct sequencing of polymerase chain reaction (PCR) products from the intact genome DNA was used to verify junction sequences between fragments of the master set, to resolve gaps and to ensure a minimum twofold redundancy for each strand. DNA hairpin ends were PCR-amplified, cloned and sequenced. Capital letters on the maps denote *Hind*III restriction enzyme fragments.

TABLE 2 VAR-BSH potential host-interactive proteins specified by selected ORFs within DNA terminal regions

Protein*		No. of residues†		Identities‡	Homologue or putative role§
VAR	VAC	VAR	VAC		
Left terminal					
D4R	C11R	140	142	89%/142	Vaccinia epidermal growth factor analogue
D6R	21.7K	242	184	96%/184	Murine rpt1 zinc-finger protein (A30891)
D8L	8.6K	452	62	95%/62	Human ankyrin (S08275)(cowpox host-range protein)
D8L	8.8K	452	69	93%/69	Human ankyrin (S08275)
D8L	13.6K	452	114	83%/114	Human ankyrin (S08275)
D11L	C7L	150	150	99%/150	Vaccinia host-range protein
D15L	C2L	263	263	95%263	Vaccinia complement-binding protein
P1L	N1L	117	117	94%/117	Vaccinia 'virokine' secreted protein
O1L	M1L	446	472	98%/447	Human ankyrin (S08275)
O3L	K1L	70	284	91%/63	Vaccinia host-range protein
C1L	K1L	66	284	97%/66	Vaccinia host-range protein
C2L	K2L	373	369	94%/373	SPI (syncytia formation inhibitor)
C3L	K3L	87	88	82%/87	Vaccinia interferon resistance protein
Right terminal					
J7R	A56R	313	315	85%/317	Haemagglutinin protein (syncytia formation inhibitor)
B1R	B1R	232	300	97%/231	Vaccinia protein kinase
B5R	B4R	558	558	94%/558	Human ankyrin (S08275)
B6R	B5R	317	317	93%/316	Vaccinia envelope glycoprotein
B8R	B8R	266	272	92%/263	Human interferon-γ receptor (A31555)
B11R	B12R	104	283	80%/71	Protein kinase
B12R	38K	344	345	92%/344	SPI (leukotriene or IL-1β pathway inhibitor)
B16R	B18R	574	574	94%/574	Human ankyrin (S08275)
B17R	B19R	354	353	89%/355	IL-1 receptor-related surface antigen
B18R	B20R	787	127	95%/123	Human ankyrin (S08275)
B21R	C12L	372	353	92%/357	SPI
B22R	absent	1,897	—	—	Novel ~214K protein
G1R	B25R	585	259	79%/231	Human ankyrin (S08275)
G1R	B26R	585	103	64%/94	Human ankyrin (S08275)
G1R	B27R	585	113	89%/97	Human ankyrin (S08275)
G2R	B28R	348	122	85%/89	Human TNF receptor (A36475)

* Proteins designated by the VAR-BSH encoding ORFs were compared with cognates specified by VAC-CPN^{2,3} or VAC-WR^{12,13}; see also refs 5 and 11 for review. Proteins absent in VAC-CPN but present in VAC-WR are designated by size. † Number of amino acids predicted from the translated ORF. ‡ Per cent amino-acid identity per number of residues homologous by FASTA²³. § Homologues of vaccinia (Genbank numbers M35027, M24218, M22812) or cellular proteins (PIR accession number shown). Abbreviations: SPI, serine protease inhibitor analogue; IL, interleukin; TNF, tumour necrosis factor.

ORFs that translated to truncated proteins compared with vaccinia correlates, and VAC-CPN specified seven truncated versions of VAR-BSH proteins, indicating major protein adaptations that differentiate these viruses.

Various poxvirus proteins, often encoded in the terminal region and non-essential for virus growth in culture, may play crucial roles during natural infection to ensure virus survival in the host^{5,11}. Table 2 shows a selected group of such proteins of VAR-BSH and their marked similarity to VAC-CPN^{2,3} or VAC-WR^{12,13} analogues, supporting the notion that rather small differences augment the concerted effects of all proteins in determining the systemic or localized nature of infection by these viruses. The host-interactive proteins (reviewed in refs 5 and 11) shown in Table 2 include homologues of epidermal growth factor, probably for increasing virus-susceptible cell numbers around infectious centres; a zinc-finger protein and protein kinases, which have the potential to influence viral gene expression and macromolecular synthesis; proteins (for example, haemagglutinin protein and serine protease inhibitor analogues) that potentially influence viral spread by altering infected cell membranes; and cellular erythrocyte ankyrin-related proteins that may specify tissue tropism. Proteins that could alter specific host defences are also encoded, including cytokine-response modifiers (cytokine receptor cognates), inhibitors of inflammation (leukotriene and interleukin-1 convertase inhibitor), and complement and interferon modulators.

The most novel VAR-BSH protein is specified by ORF B22R (ORF nomenclature follows that of VAC-CPN^{2,3}), which shows poor homology to current database proteins. This protein (*M*, 214K) has six possible transmembrane domains, suggesting that it may locate to virion or infected-cell membranes, and thereby profoundly impact infection. VAR-BSH G2R encodes a human tumour necrosis factor (TNF) receptor homologue; the corresponding VAC-CPN ITR ORF, B28R (duplicated as C22L) specifies a truncated variant with an incomplete TNF-binding domain. The VAR-BSH D6R product contains a complete zinc-finger motif not found in a truncated 21.7K correlate of VAC-WR¹² (absent in VAC-CPN). VAR-BSH C3L encodes a variant (82% identity) of the interferon-resistance mediator of VAC-CPN K3L¹⁴. Together with various proteins related to cellular erythrocyte ankyrin, which possibly contribute to tissue tropism, VAR-BSH D11L specifies a homologue of the VAC-CPN C7L product known to influence viral infectivity for certain cell lines¹⁵; but D11L includes an Arg-Gly-Asp cell-adhesion motif¹⁶ which could markedly influence tissue tropism. Finally, not listed, is VAR-BSH J5.5R (45 residues with partial homology to VAC-CPN A55R), an analogue of vascular endothelial growth factor, which has counterparts in the genus *Parapoxvirus* (A. J. Robinson, personal communication), suggesting a virulence determinant stemming from a common poxvirus ancestor.

Comparison of VAR-BSH with VAC-CPN sequences does not allow determination of variola virus origins or explain why variola was so virulent and had such a strict human host range. Destruction of all remaining variola laboratory samples has been recommended by the World Health Organization as the final step in smallpox eradication^{17,18}, and if this plan proceeds, further examination of gene products by replicating live virus will cease. With virtually no data on authentic proteins, we can only speculate on correlations with other poxviruses and assume that putative VAR-BSH ORFs and ORFs of other variola strains recently reported^{9,19-22} are expressed. Nevertheless, the terminal region features of VAR-BSH highlighted here and further described in context of the entire VAR-BSH genetic composition (R. F. M. *et al.*, manuscript submitted) should prove valuable to future investigation of viral pathogenesis. □

Received 30 July; accepted 25 October 1993.

1. Fenner, F. *et al.* *Smallpox and its Eradication* (World Health Organization, Geneva, 1988).
2. Goebel, S. J. *et al.* *Virology* **179**, 247-266 (1990).

3. Johnson, G. P., Goebel, S. J. & Paoletti, E. *Virology* **196**, 381–401 (1993).
4. Fenner, F., Wittek, R. & Dumbell, K. *The Orthopoxviruses* (Academic, San Diego, 1989).
5. Buller, R. M. L. & Palumbo, G. J. *Microbiol. Rev.* **55**, 80–122 (1991).
6. Moss, B. *Curr. Top. Microbiol. Immun.* **163**, 41–70 (1990).
7. Esposito, J. J. & Knight, J. C. *Virology* **143**, 230–251 (1985).
8. Esposito, J. J. in *Classification and Nomenclature of Viruses Arch. Virol. Suppl.* 2 (eds Francki, R. I. B. et al.) 91–102 (Springer, New York, 1991).
9. Aguado, B., Selmes, I. P. & Smith, G. L. *J. gen. Virol.* **73**, 2887–2902 (1992).
10. Smith, G. L., Chan, Y. S. & Howard, S. T. *J. gen. Virol.* **72**, 1349–1376 (1991).
11. Smith, G. L. *J. gen. Virol.* **74**, 1725–1740 (1993).
12. Kotwal, G. J. & Moss, B. *Virology* **167**, 524–537 (1988).
13. Kotwal, G. J. & Moss, B. *J. Virol.* **63**, 600–606 (1989).
14. Beattie, E., Tartaglia, J. & Paoletti, E. *Virology* **183**, 419–422 (1991).
15. Perkus, M. E. et al. *Virology* **179**, 276–286 (1990).
16. Rouslahti, E. & Perschbacher, M. D. *Cell* **44**, 517–518 (1986).
17. Mahy, B. W. J., Esposito, J. J. & Venter, J. C. *ASM News* **57**, 577–580 (1991).
18. Mahy, B. W. J. & Howard, J. C. *Rev. med. Virol.* **3**, 131–135 (1993).
19. Shchelkunov, S. N. et al. *Virus Res.* **27**, 25–35 (1993).
20. Shchelkunov, S. N., Blinov, V. M. & Sandakhchiev, L. *FEBS Lett.* **319**, 80–83 (1993).
21. Shchelkunov, S. N., Blinov, V. M. & Sandakhchiev, L. *FEBS Lett.* **319**, 163–165 (1993).
22. Shchelkunov, S. N. et al. *Doklady Akademii Nauk* **328**, 629–632 (1993).
23. Pearson, W. R. & Lipman, D. J. *Proc. natn. Acad. Sci. U.S.A.* **85**, 2444–2448 (1988).
24. Henikoff, S. *Gene* **28**, 351–359 (1984).
25. Sambrook, J., Fritsch, E. F. & Maniatis, T. *Molecular Cloning: A Laboratory Manual* 2nd edn (Cold Spring Harbor Laboratory Press, New York, 1989).
26. Martin-Gallardo, A. et al. *Nat. Genet.* **1**, 34–39 (1992).
27. Gocayne, J. D. et al. *Proc. natn. Acad. Sci. U.S.A.* **84**, 8296–8300 (1987).

ACKNOWLEDGEMENTS. R.F.M. and J.J.E. are joint first authors of this letter. We thank A. Martin-Gallardo, D. McGeoch, W. R. McCombie and P. Pellett for advice on designing sequencing strategies; H. Walls and J. House for administrative assistance; the CDC Core Facility and the NINDS and TIGR Oligonucleotide Facilities for primer synthesis; the TIGR Computational Genomics Facility and the CDC Core Facility for computational support; and Applied Biosystems, Inc. for their INHERIT sequence assembly and analysis system. This project was partly funded by the National Institute of Allergy and Infectious Disease, NIH, and The Rockefeller Foundation.

Crystal structure of porcine ribonuclease inhibitor, a protein with leucine-rich repeats

Bostjan Kobe & Johann Deisenhofer

Howard Hughes Medical Institute and Department of Biochemistry, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75235-9050, USA

RIBONUCLEASE inhibitor is a cytoplasmic protein that tightly binds and inhibits ribonucleases of the pancreatic ribonuclease superfamily¹. The primary sequence of this inhibitor contains leucine-rich repeats² (LRRs); these motifs are present in many proteins that participate in protein–protein interactions and have different functions and cellular locations. *In vivo*, ribonuclease inhibitor may have a role in the regulation of RNA turnover in mammalian cells³ and in angiogenesis⁴. To define the structural features of LRR proteins and to understand better the nature of the tight interaction of ribonuclease inhibitor with ribonucleases, we have determined the crystal structure of the porcine inhibitor. To our knowledge, this is the first three-dimensional structure of a protein containing LRRs and represents a new class of α/β protein fold. Individual repeats constitute β - α structural units that probably also occur in other proteins containing LRRs. The non-globular shape of the structure and the exposed face of the parallel β -sheet may explain why LRRs are used to achieve strong protein–protein interactions. A possible ribonuclease-binding region incorporates the surface formed by the parallel β -sheet and the β α loops.

The crystal structure was solved by multiple isomorphous replacement (MIR) using ten heavy-atom derivatives (Table 1) and refined at 2.5 Å resolution (Fig. 1a). The current atomic model of porcine ribonuclease inhibitor (RI) contains all 456 residues and no solvent molecules. Porcine RI is built of 15 tandem homologous repeats of 29 (A-type) and 28 (B-type) amino acids which alternate in the sequence, with only two short non-homologous flanking sequences at the termini². In the three-dimensional structure (Fig. 1), individual LRRs correspond to right-handed β - α structural units, similar to those found in

many α/β proteins, but are arranged differently from other known α/β structures. The β -strand and the α -helix are roughly parallel in individual β - α units, and the strands and the helices are all aligned approximately parallel to a common axis. This axis is the symmetry axis of the molecule; a repeat can be superimposed on a neighbouring repeat by a rotation of about 17° around this axis. The resulting structure has the shape of a horseshoe, with overall dimensions 70 × 62 × 32 Å (Fig. 1). With the flanking sequences adopting a similar conformation as the repeats, 17 β -strands form a curved parallel β -sheet on the inner circumference of the horseshoe, and 16 helices align on the outer circumference. One face of the parallel β -sheet is exposed to the solvent, unlike in other α/β structures. Because of the curvature of the sheet, the side chains efficiently protect the backbone of the sheet from contact with solvent. With the exception of the shorter N-terminal helix of eight residues, all helices are between 11 and 13 residues long. The average angle between the neighbouring helices is 12.7 ± 5.3°. The β -strands (as defined in the program DSSP⁵) comprise only three residues, with the exception of the two N-terminal strands of five residues. Each strand forms six hydrogen bonds with the neighbouring two strands in the sheet. Both the loops connecting the β -strands with the α -helices (β α loops) and the α β loops on the opposite side contain between 4 and 9 amino acids.

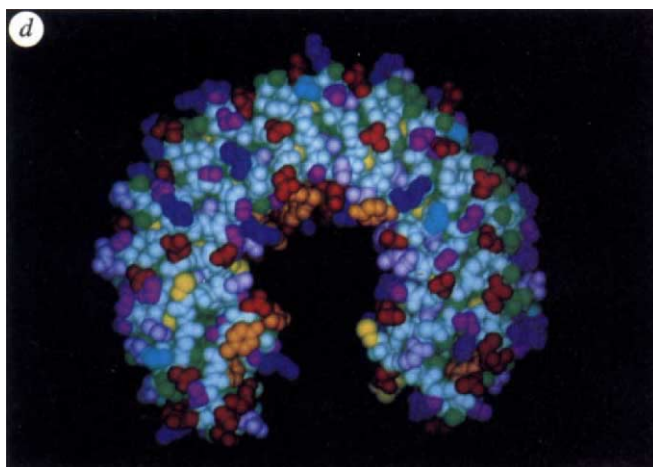
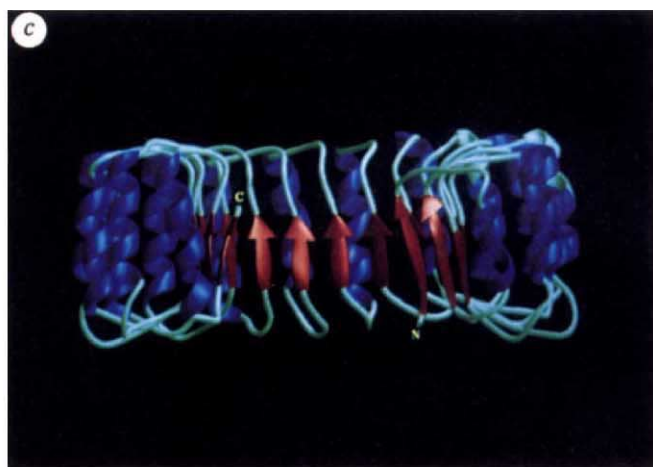
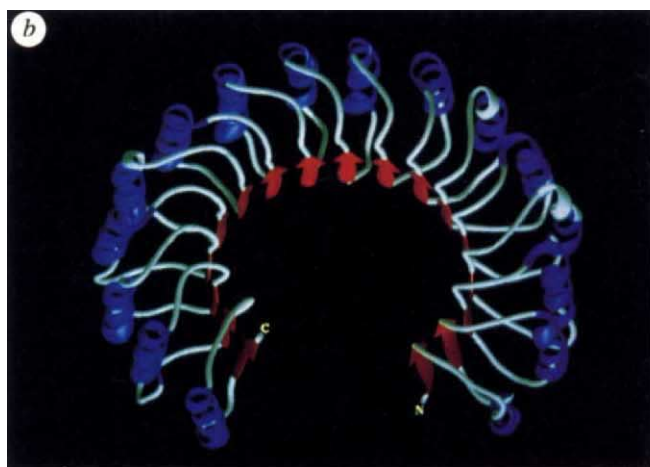
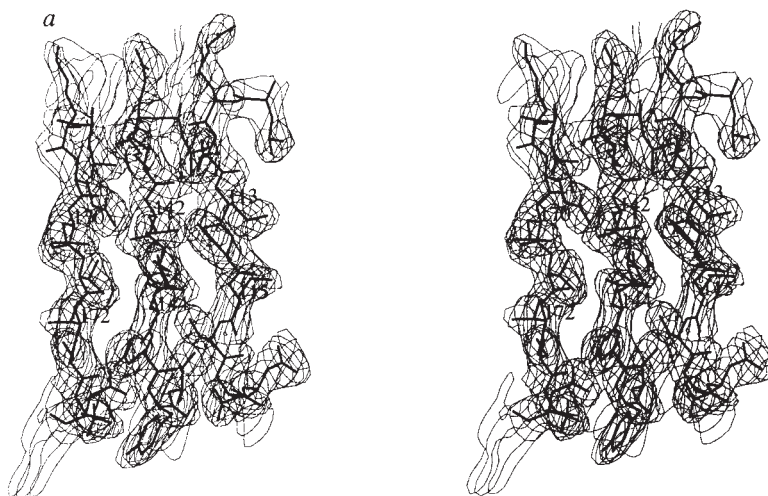
The β -sheet lacks the typical twist (Fig. 1c), which may be due to the shortness of the β -strands and the presence of favourable side-chain stacking interactions when the sheet is flat. In the absence of the twist, the curvature of the horseshoe is determined solely by the difference in the distances between the strands and the helices. The average distance is 9.32 ± 1.10 Å between neighbouring helices and 4.84 ± 0.12 Å between neighbouring β -strands (the distances were calculated as an average distance of 11 equivalent atoms in neighbouring helices and 3 equivalent atoms in neighbouring β -strands; the central 14 helices and strands were used in the calculation). In parallel β -barrel structures, the β -sheet twist and the requirement to exclude solvent from the barrel interior are the major factors that constrain the number of strands to eight⁶. The lack of both these factors in RI results in a different arrangement of the β - α structural units, although the connections between these structural units are the same as in parallel β -barrels.

Porcine RI contains 30 cysteine residues; all are in the free thiol form in the active inhibitor in the crystal. When the reducing agent is removed, the inhibitor undergoes a conformational change and becomes inactive by an 'all-or-none' mechanism whereby all cysteines form disulphide bridges⁷. In the inactive protein, a disulphide bridge formed between residues 261 and 268 (ref. 7). In the crystal structure, the Ca atoms of these residues are 11.4 Å apart; this confirms that a major conformational change is required to form the disulphide bridges. The structure of the reduced form of RI does not indicate which cysteine pairs are likely to form disulphide bridges in the oxidized form.

On the basis of the three-dimensional structure, we have aligned the repeats in RI in a way different from the original proposal² so that the elements of secondary structure and the core conserved region of the repeats are not split (Fig. 2a). The first and the last repeat in the structure deviate from the consensus alignment. The average r.m.s. deviations for the backbone atoms of the central seven A-type and B-type repeats are 0.90 ± 0.26 and 1.00 ± 0.23 Å, respectively (Fig. 2b). The corresponding value when comparing the A-type with the first 28 residues of the B-type is 1.68 ± 0.23 Å.

The residues of the consensus sequence of the repeats play clear structural roles (Fig. 2c, d and e). The side chains of leucines and of other aliphatic residues (positions 2, 5, 7, 12, 17, 20, 23 and 24) form the hydrophobic core of the protein. Cysteines at position 17 in the A-type repeats are also a part of the hydrophobic core. The conserved cysteines and asparagines at position 10 are buried, too; the side chains form hydrogen

FIG. 1 Structure of porcine RI. *a*, Stereo view of the electron density in the β -sheet region of the protein, calculated with model-derived phases and coefficients $2|F_{obs}| - |F_{calc}|$ for data between 40 and 2.5 Å, contoured at one standard deviation and plotted with a program written by D. Xia. Superimposed is the current model of RI. Ladders of conserved hydrophobic residues can be observed. *b*, Schematic diagram of the three-dimensional structure of porcine RI, drawn with the program RIBBONS³⁹. The β -sheet is red, α -helices are blue and the rest is green. The 3_{10} helices are the green parts of the helices. *c*, As in *b*, but rotated by 90° around the x-axis. *d*, Space-filling model of RI in a view similar to *a*, drawn with the program INSIGHT (Biosym). Protein backbone (N, C α , C, O atoms) is coloured white and side chains are coloured by amino acid (Ala, Leu, Ile, Val, green; Phe, Trp, Tyr, orange; Cys, Met, yellow; His, Arg, Lys, blue; Asp, Glu, red; Asn, Gln, pink; Ser, Thr, purple; Pro, cyan).



bonds with the backbone. In the B-type consensus, glycine at position 13 seems to be a helix-initiation signal and proline at position 27 a helix-termination signal.

On the surface of the protein, a ladder is formed by alternating negatively charged side chains of residues at position 3 in A-type repeats and positively charged side chains of residues at position 3 in B-type repeats. A similar ladder is formed by side chains of residues at positions 4; between the consensus glutamates in the B-type repeats there are either positively charged amino acids, or serines or threonines in the A-type repeats. Yet another hydrogen-bonding network is constructed by side chains

of threonines at position 13 in A-type repeats and aspartates at position 14 in B-type repeats.

Among over thirty known LRR proteins are the receptors for gonadotrophins⁸ and neurotrophins⁹, the cell adhesion molecules *Toll*¹⁰, chaoptin¹¹ and platelet GIIb¹², the extracellular-matrix-binding glycoproteins decorin¹³ and fibromodulin¹⁴, the regulatory domains or subunits of the enzymes adenylyl cyclase¹⁵ and carboxypeptidase N¹⁶, bacterial virulence factors¹⁷, molecules involved in splicing¹⁸ and DNA repair¹⁹, and many others. The residues that are conserved throughout this LRR superfamily all cluster around the β -strand region (Fig. 2a). Usually the repeats are between 20 and 29 residues long. Porcine RI contains not only 28- and 29-residue repeats, but also a 25-residue repeat formed by the N-terminal segment. The helix is shorter (only eight residues) than in the other repeats in RI and the $\alpha\alpha$ turn comes earlier ($i+1$ and $i+2$ are at positions 23 and 24, respectively, in the repeat). When repeats are even shorter and when there are prolines in the C-terminal part of the repeat, a more extended structure might substitute for the helix. An example of such a structure is the β -helix, a structural motif found in pectate lyase C (ref. 20). A perfect turn of a β -helix without loops is 22 residues long. The β -helix structure has several features in common with the structure of RI, including a parallel β -sheet with one face of the sheet exposed to solvent and a ladder of asparagine residues forming hydrogen bonds with the backbone at one end of β -strands.

In RI, the termini would collide if more than 21 repeats were present. In such a case, a clash between termini could be avoided by a twist in the sheet so that the repeats proceeded in a helical fashion; alternatively, smaller numbers of repeats could constitute separate domains that would be linked by segments adopt-

TABLE 1 Structure determination and refinement statistics

Data collection statistics

Derivative	Soaking		Unit cell (Å)		Resolution (Å)	Observations		Completeness (>2σ)	R_{merge}^* (%: >3σ)	$\langle \Delta F/F \rangle^\dagger$ (10 ⁻⁴ Å)
	c (mM)	t	a	c		Total	Unique			
Native-1 ‡	—	—	136.2	84.1	20–3.1	90,803	13,846	0.829	7.64	—
Native-2 §	—	—	134.8	83.6	20–2.5	147,432	25,809	0.713	7.10	—
KAu(CN) ₂	10	18 h	137.2	84.5	20–3.5	38,707	9,706	0.804	8.69	0.212
KAuCl ₄ -1	1	40 h	136.1	83.9	20–3.5	32,639	9,536	0.603	9.53	0.103
KAuCl ₄ -2	1	5 d	136.1	83.9	20–3.5	40,199	9,659	0.660	8.47	0.092
pHMB-1	1	26.5 h	136.4	83.9	20–3.5	36,987	7,893	0.639	8.47	0.099
pHMB-2	5	2 d	136.0	83.9	20–3.5	10,558	6,256	0.495	9.38	0.219
pHMB-3	5	4 d	136.9	84.4	20–3.5	19,065	8,993	0.534	9.48	0.211
TAMM	1	3 d	136.2	84.0	20–3.5	39,473	9,537	0.697	7.78	0.085
K ₂ IrCl ₆ -1	10	8.5 d	134.9	83.2	20–3.5	20,509	7,154	0.513	7.88	0.222
K ₂ IrCl ₆ -2	5	72 h	136.7	84.0	20–3.5	41,296	9,677	0.742	8.31	0.084
KReO ₄	5	3 d	136.2	83.9	20–3.5	37,081	9,632	0.714	8.79	0.069

Heavy-atom parameters

Derivative	$f_H/\text{res}\#$	R_{cullis}^{**}	Sites			Relative occupancy	$B(\text{\AA}^2)$
			x	y	z		
KAu(CN) ₂	1.71	0.74	0.0298	0.3322	0.1250	1.93	47.5
			0.1499	0.1963	0.1590	1.43	52.9
			0.3083	0.1019	0.1522	1.33	58.5
KAuCl ₄ -1	1.03	0.86	0.1501	0.2000	0.1685	0.84	44.1
			0.1505	0.2015	0.1687	0.50	22.7
			0.3153	0.1080	0.1491	0.50	21.3
pHMB-1	1.04	0.90	0.4670	0.1477	0.4448	0.53	42.5
			0.3141	0.1077	0.1509	1.39	38.4
			0.3151	0.1072	0.1527	1.06	26.8
pHMB-2	0.67	0.94	0.1494	0.2003	0.1608	0.75	48.7
			0.4664	0.1480	0.4415	1.10	50.7
			0.0342	0.4148	0.3390	0.65	48.9
pHMB-3	0.88	0.99	0.1481	0.2089	0.1866	1.22	26.9
			0.0257	0.2766	0.2190	0.50	102.7
			0.1385	0.2996	0.4570	0.37	48.9
TAMM	0.88	0.94	0.0420	0.3030	0.2640	0.35	61.2
			0.1539	0.2035	0.1701	0.26	3.2
K ₂ IrCl ₆ -1	0.72	0.91					
K ₂ IrCl ₆ -2	0.64	0.96					
KReO ₄	0.64	0.97					

Refinement

Resolution (Å)	40–2.5
$R_{\text{cryst}}^{\dagger\dagger}$ (%)	18.2
$R_{\text{free}}^{\dagger\dagger}$ (%)	26.9
Number of reflections used (>2σ)	18,528
Total number of non-hydrogen atoms	3,411
R.m.s. bond lengths deviations (Å)	0.012
R.m.s. bond angles deviations (°)	1.748

Crystals of porcine RI have the symmetry of the tetragonal space group I4 (refs 28, 29). The phase problem was solved by the method of MIR. A stabilizing solution containing 1.8 M lithium sulphate, 20 mM HEPES buffer, pH 7, and 20 mM dithiothreitol (DTT) was used for all soaks with heavy-atom reagents. A reference native data set to 3.1 Å resolution (native-1) was collected using two crystals that were soaked in the stabilizing solution for two days. Data for all heavy-atom derivatives were collected from individual crystals. Multiwire area detectors of Xuong–Hamlin design³⁰ with CuKα radiation from a rotating anode were used for data collections. Data were processed with the software by Howard *et al.*³¹ and PROTEIN³². Heavy-atom binding was analysed by vector verification in the Patterson map; the most useful criterion for finding a specific binding site was a correlation of vector verification solutions at two non-overlapping resolution ranges (10–7 and 7–5 Å were used). Cross-difference Fourier maps were used to confirm the sites. Heavy-atom parameters were refined using the least-squares algorithms in PROTEIN. The overall figure of merit for data between 20 and 3.5 Å was 0.50. Although the self rotation functions indicated that the asymmetric unit probably contained two molecules^{28,29}, the electron density maps and measurements of crystal density in a gradient of Ficoll 400 (ref. 33) (prepared in 1.8 M ammonium sulphate to prevent crystals from dissolving) indicated that only one RI molecule was present. The non-interpretable electron density calculated from the MIR phases was modified by solvent flattening³⁴, yielding an interpretable map which was further improved by re-refining the heavy-atom parameters with the phases obtained after solvent flattening and repeating the solvent-flattening procedure. The initial model built using the graphics program O (ref. 35) in the 20–3.5-Å electron density map contained all 456 residues. The N- to C-terminal direction of the polypeptide was confirmed by inspection of the heavy-metal binding sites. The main-chain and side-chain atoms were added to the initial Ca model using the fragment library; fragments of 5 residues were used mostly, except in the α helical regions where the length of the entire helix determined the length of the fragment. Program package XPLOR³⁶ was used for refinement. The initial model had an R_{cryst} of 0.415 for data between 8 and 3.1 Å. Positional refinement, overall temperature factor refinement and manual rebuilding resulted in an R_{cryst} of 0.227. Refinement was then continued with data collected from crystals grown in ammonium sulphate (native-2)²⁸. The initial R_{cryst} was 0.395 for data between 10 and 4 Å, and dropped to 0.330 after rigid-body refinement. The model was further refined using simulated annealing. At first, data from 8–2.5 Å were used; later, a bulk solvent mask was introduced and lower-resolution data included. Only data with $F > 2\sigma(F)$ were used in the refinement; completeness in the highest resolution shell between 2.61 and 2.5 Å is 45.6%. Individual B-factors were refined as isotropic and restrained. The present model contains no water molecules; no likely water molecule positions were detected in the interior of the protein. All non-glycine residues have (ϕ , ψ) angles within allowed hard-sphere limits. The mean coordinate error is estimated to be 0.26 Å based on a Luzzati plot³⁷, and 0.41 Å based on the SIGMA method³⁸. The temperature factors of main-chain atoms exceed 60 Å² in residues 1–15, 135–136 and 447–451. The coordinates will be deposited in the Brookhaven Protein Data Bank.

* $R_{\text{merge}} = \sum_{hkl} (\sum_i |I_{hkl,i} - \langle I_{hkl} \rangle|) / \sum_{hkl} \langle I_{hkl} \rangle$, where $I_{hkl,i}$ is the intensity of an individual measurement of the reflection with Miller indices h , k and l , and $\langle I_{hkl} \rangle$ is the mean intensity of that reflection.

† $\langle \Delta F/F \rangle$ (mean fractional isomorphous difference) = $(\sum_{hkl} (|F_{hkl,P}| - |F_{hkl,PH}|)) / (\sum_{hkl} |F_{hkl,P}|)$, where $F_{hkl,P}$ is the structure factor of the reflection with Miller indices h , k and l from the native-1 data set and $F_{hkl,PH}$ is the structure factor for the corresponding reflection from the derivative data set. Only reflections with $F > 3\sigma(F)$ were included in the calculation.

‡ Native-1 is the data set collected using two crystals soaked for two days in the stabilizing solution containing lithium sulphate and HEPES buffer.

§ Native-2 is the data set collected using three crystals grown in the presence of ammonium sulphate and phosphate buffer²⁸.

|| pHMB is *p*-hydroxymercuribenzoate.

* TAMM is tetrakis(acetoxymethyl)mercuric methanide.

R.m.s. $f_H/\text{residual}$ represents the phasing power of a derivative and is the ratio of the amplitude of the r.m.s. heavy-atom scattering factor to the r.m.s. lack of closure.

** $R_{\text{cullis}} = \sum_{hkl} (|F_{hkl,H}| - |F_{hkl,P}|) / \sum_{hkl} |F_{hkl,H}|$, where $F_{hkl,H}$ represents the calculated heavy-atom structure factor contribution. The summation is valid only for centric reflections.

†† $R_{\text{cryst}} = \sum_{hkl} (|F_{\text{obs}}| - |F_{\text{calc}}|) / \sum_{hkl} |F_{\text{obs}}|$, where $|F_{\text{obs}}|$ and $|F_{\text{calc}}|$ are the observed and calculated structure factor amplitudes. Ten per cent of reflections were omitted in refinement and used for cross-validation.

‡‡ R_{free} is equivalent to R_{cryst} , but computed for a test set of 10% reflections that were omitted in the refinement process.

Porcine RI inhibits ribonuclease (RNase) A, with an extremely low inhibitory constant (K_i) of 6.7×10^{-14} M²². Analysis of the regions of RNase A and angiogenin that interact

Abstract

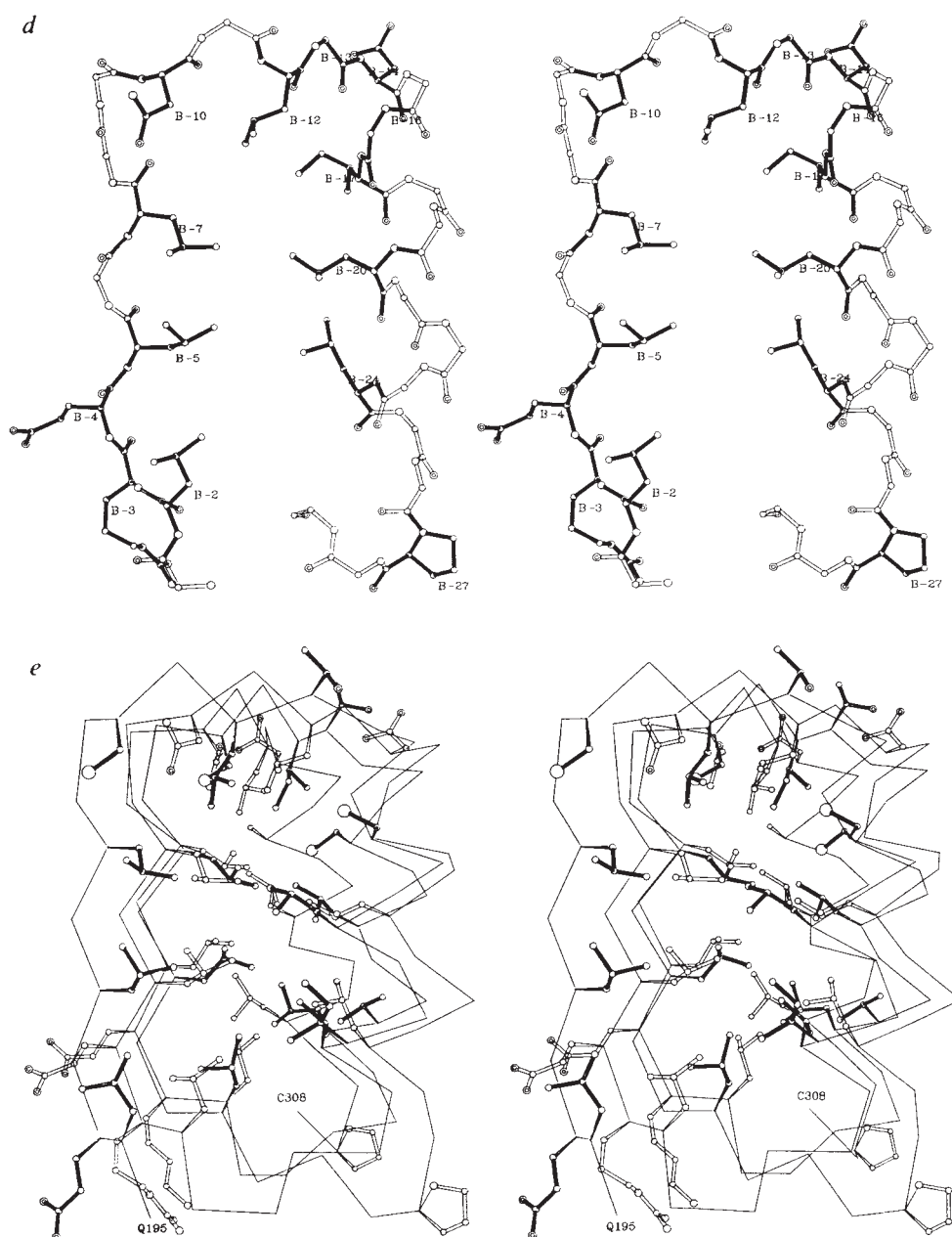
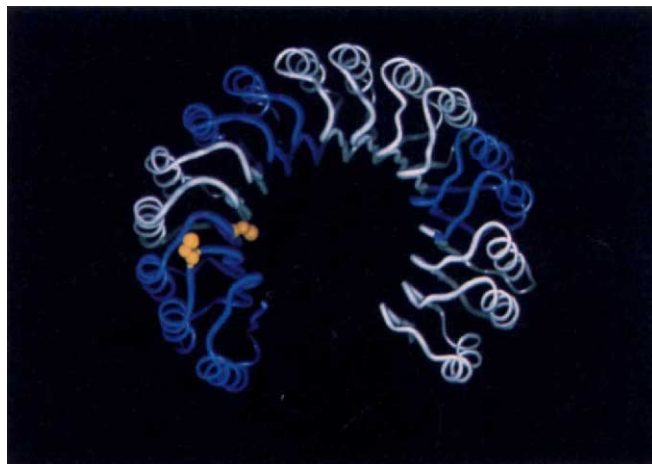


FIG. 3 Ribonuclease binding. In the ribbon diagram of RI drawn with the program INSIGHT, the parts of the inhibitor that can be removed without destroying the inhibitory activity^{23,24} are coloured white; the side chains of the two cysteines that are protected from labelling in the presence of RNase A²⁶ are shown in yellow. When internal repeats are deleted from the sequence as pairs of A- and B-type repeats²³, the protein can probably fold in a conformation similar to intact RI, because most residues interacting with neighbouring repeats are conserved in each repeat. The conformation of the ribonuclease binding site must be altered in mutants with deleted internal repeats, but the part of the structure that has not been perturbed could still bind RNase in the same manner as the intact inhibitor. The rest may not be involved in binding or may interact in new ways. The resulting affinity should therefore be weaker, as was indeed observed: the K_i values for RNase A of the human RI deletion mutants $\Delta 144-257$ and $\Delta 315-371$ are reduced 2,500-fold and 630-fold, respectively²⁴.



as would be expected for the binding site for the basic ribonuclease molecule. The diameter of the cavity formed by the β -sheet is between 18 and 25 Å, with the shortest distance between the terminal repeats of only 13 Å (Fig. 1d). RNase A has dimensions of $20 \times 25 \times 45$ Å and no conformational changes upon binding have been reported. Therefore only a part of the ribonuclease molecule could bind in the cavity formed by the β -sheet. Because most tertiary interactions stabilizing the structure of RI occur between successive β - α building blocks, the two ends of the horseshoe may be able to move substantially relative to each other in solution. The resulting elasticity of the structure may be important in ribonuclease binding. □

Received 11 August; accepted 15 November 1993.

1. Lee, F. S. & Vallee, B. L. *Prog. Nucleic Acids Res. molec. Biol.* **44**, 1–30 (1993).
2. Hofsteenge, J. et al. *Biochemistry* **27**, 8537–8544 (1988).
3. Quirin-Stricker, C., Gross, M. & Mandel, P. *Biochim. biophys. Acta* **159**, 75–80 (1968).
4. Shapiro, R. & Vallee, B. L. *Proc. natn. Acad. Sci. U.S.A.* **84**, 2238–2241 (1987).
5. Kabsch, W. & Sander, C. *Biopolymers* **22**, 2577–2637 (1983).
6. Lasters, et al. *Proc. natn. Acad. Sci. U.S.A.* **85**, 3338–3342 (1988).
7. Fominaya, J. M. & Hofsteenge, J. *J. biol. Chem.* **267**, 24655–24660 (1992).
8. McFarland, K. C. et al. *Science* **245**, 494–499 (1989).
9. Schneider, R. & Schweiger, M. *Oncogene* **6**, 1807–1811 (1991).
10. Hashimoto, C., Hudson, K. L. & Anderson, K. V. *Cell* **52**, 269–279 (1988).
11. Reinke, R. et al. *Cell* **52**, 291–301 (1988).
12. Lopez, J. A. et al. *Proc. natn. Acad. Sci. U.S.A.* **84**, 5615–5619 (1987).
13. Krusius, T. & Ruoslahti, E. *Proc. natn. Acad. Sci. U.S.A.* **83**, 7683–7687 (1986).
14. Oldberg, Å. et al. *EMBO J.* **8**, 2601–2604 (1989).
15. Kataoka, T., Broek, D. & Wigler, M. *Cell* **43**, 493–505 (1985).
16. Tan, F. et al. *J. biol. Chem.* **265**, 13–19 (1990).
17. Leung, K. Y. & Straile, S. C. *J. Bact.* **171**, 4623–4632 (1989).
18. Fresco, L. D., Harper, D. S. & Keene, J. D. *Molec. cell. Biol.* **11**, 1578–1589 (1991).
19. Schneider, R. & Schweiger, M. *FEBS Lett.* **283**, 203–206 (1991).
20. Yoder, M. D., Keen, N. T. & Jurnak, F. *Science* **260**, 1503–1507 (1993).
21. Young, D. et al. *Proc. natn. Acad. Sci. U.S.A.* **86**, 7989–7993 (1989).
22. Vicentini, A. M. et al. *Biochemistry* **29**, 8827–8834 (1990).
23. Lee, F. S. & Vallee, B. L. *Proc. natn. Acad. Sci. U.S.A.* **87**, 1879–1883 (1990).
24. Lee, F. S. & Vallee, B. L. *Biochemistry* **29**, 6633–6638 (1990).
25. Hofsteenge, J., Vicentini, A. & Stone, S. R. *Biochem. J.* **275**, 541–543 (1991).
26. Hofsteenge, J., Servis, C. & Stone, S. R. *J. biol. Chem.* **266**, 24198–24204 (1991).
27. Nicholls, A. & Honig, B. *J. comp. Chem.* **12**, 435–445 (1991).
28. Kobe, B. & Deisenhofer, J. *J. molec. Biol.* **231**, 137–140 (1993).
29. Neumann, U. et al. *J. molec. Biol.* **231**, 505–508 (1993).
30. Hamlin, R. *Meth. Enzym.* **114**, 416–452 (1985).
31. Howard, A. J., Nielsen, C. & Xuong, N. H. *Meth. Enzym.* **114**, 452–472 (1985).
32. Steigemann, W. *PROTEIN: A Program System for the Crystal Structure Analysis of Proteins* (Max-Planck-Institut für Biochemie, Martinsried, Germany, 1992).
33. Bode, W. & Schirmer, T. *Biol. Chem. Hoppe-Seyler* **366**, 287–295 (1985).
34. Wang, B.-C. *Meth. Enzym.* **115**, 90–112 (1985).
35. Jones, T. A., Bergdoll, M. & Kjeldgaard, M. in *Crystallographic and Modeling Methods in Molecular Design* (eds Bugg, C. E. & Ealick, S. E.) 189–195 (Springer, New York, 1990).
36. Brünger, A. T., Kuriyan, J. & Karplus, M. *Science* **235**, 458–460 (1987).
37. Luzzati, P. V. *Acta crystallogr.* **5**, 802–810 (1952).
38. Read, R. J. *Acta crystallogr.* **A42**, 140–149 (1986).
39. Carson, M. J. *molec. Graphics* **5**, 105–106 (1987).
40. Wilmot, C. M. & Thornton, J. M. *Protein Engng* **3**, 479–493 (1990).

ACKNOWLEDGEMENTS. We thank K. G. Ravichandran, C. Hasemann, H.-W. Park, B. Goldsmith, D. Crawford and B. Beutler for encouragement and discussions; D. Coleman for help with X-ray equipment; D. Diggs and K. Watkins for help with programs; D. Staber for help with the manuscript; and K. Fischer-Lindahl for review and suggestions.

CORRECTION

A protein kinase homologue controls phosphorylation of ganciclovir in human cytomegalovirus-infected cells

V. Sullivan, C. L. Talarico, S. C. Stanat, M. Davis, D. M. Coen & K. K. Biron

Nature **358**, 162–164 (1992).

FIGURE 3 of this letter contains an error in the numbering of the amino-acid residues of the HCMV UL97 protein. The 4-amino-acid deletion in UL97 should comprise residues 590–593, and not residues 638–641 as indicated in the figure. □

GUIDE TO AUTHORS

PLEASE follow these guidelines so that your manuscript may be handled expeditiously.

Nature is an international journal covering all the sciences. Contributors should therefore bear in mind those readers who work in other fields and those for whom English is a second language, and write clearly and simply, avoiding unnecessary technical terminology. Space in the journal is limited, making competition for publication severe. Brevity is highly valued. One printed page of *Nature*, without display items, contains about 1,300 words.

Manuscripts are selected for publication according to editorial assessment of their suitability and reports from independent referees. They can be sent to London or Washington and should be addressed to the Editor. Manuscripts may be dealt with in either office, depending on the subject matter, and will where necessary be sent between offices by overnight courier. High priority cannot be given to presubmission enquiries; in urgent cases they can be made in the form of a one-page fax. All manuscripts are acknowledged on receipt but fewer than half are sent for review. Those that are not reviewed are returned as rapidly as possible so that they may be submitted elsewhere without delay. Contributors may suggest reviewers; limited requests for the exclusion of specific reviewers are usually heeded. Manuscripts are usually sent to two or three reviewers, chosen for their expertise rather than their geographical location. Manuscripts accepted for publication are processed from the London office.

Nature requests authors to deposit sequence and crystallographic data in the databases that exist for this purpose.

Once a manuscript is accepted for publication, contributors will receive proofs in about 4 weeks. *Nature's* staff will edit manuscripts with a view to brevity and clarity, so contributors should check proofs carefully. Manuscripts are generally published 2–3 weeks after receipt of corrected proofs. *Nature* does not exact page charges. Contributors receive a reprint order form with their proofs; reprint orders are processed after the manuscript is published and payment received.

Categories of paper

Review Articles survey recent developments in a field. Most are commissioned but suggestions are welcome in the form of a one-page synopsis addressed to the Reviews Coordinator. Length is negotiable in advance.

Progress articles review particularly topical developments for a nonspecialist readership. They do not exceed 4 pages in length. Suggestions may be made to the Reviews Coordinator in the form of a brief synopsis.

Articles are research reports whose conclusions are of general interest and which are sufficiently rounded to be a substantial advance in understanding. They should not have more than 3,000 words of text (not including figure legends) or more than six display items and should not occupy more than five pages of *Nature*.

Articles start with a heading of 50–80 words written to advertise their content in general terms, to which editors will pay particular attention. The heading does not usually contain numbers, abbreviations or measurements. The introduction to the study is contained in the first two or three paragraphs of the article, which also briefly summarize its results and implications. Articles have fewer than 50 references and may contain a few short subheadings.

Letters to Nature are short reports of outstanding novel findings whose implications are general and important enough to be of interest to those outside the field. Letters should have 1,000 or fewer words of text and four or fewer display items. The first paragraph describes, in not more than 150 words and without the use of abbreviations, the background, rationale and chief conclusions of the study for the particular benefit of non-specialist readers. Letters do not have subheadings and contain fewer than 30 references.

Commentary articles deal with issues in, or arising from, research that are also of interest to readers outside research.

News and Views articles inform nonspecialist readers about new scientific advances, sometimes in the form of a conference report. Most are commissioned but proposals can be made in advance to the News and Views Editor.

Scientific Correspondence is for discussion of topical scientific matters, including those published in *Nature*, and for miscellaneous contributions. Priority is given to letters of fewer than 500 words.

Preparation of manuscripts

All manuscripts should be typed, double-spaced, on one side of the paper only. An original and four copies are required, each accompanied by artwork. If photographs are included, five sets of originals are required; for line drawings, one set of originals and four good-quality photocopies are acceptable. Reference lists, figure legends and tables should all be on separate sheets, all of which should be double-spaced and numbered. Three copies of relevant manuscripts in press or submitted for publication elsewhere should be included with submitted manuscripts, clearly marked as such. Five copies of revised and resubmitted manuscripts, labelled with their manuscript numbers, are required, together with five copies of a letter detailing the changes made.

Titles are brief and simple. Active verbs, numerical values, abbreviations and punctuation are to be avoided. Titles should contain one or two key words for indexing purposes.

Artwork should be marked individually and clearly with the author's name and, when known, the manuscript number. Ideally, no figure should be larger than 28 by 22 cm. Figures with several parts are to be avoided and are permitted only if the parts are closely related, either experimentally or logically. Unlettered originals of photographs should be provided. Suggestions for cover illustrations, with captions and labelled with the manuscript number, are welcome. Original artwork is returned when a manuscript cannot be published.

Protein/nucleotide sequences should ideally be in the three-letter and not the single-letter code for amino acids. One column width of *Nature* can accommodate 20 amino acids or 60 base pairs.

Colour artwork. A charge of £500 per page is made as a contribution towards the cost of reproducing colour figures. Inability to pay these costs will not prevent publication of essential colour figures if the circumstances are explained. Proofs of colour artwork may be sent to contributors under separate cover from their galley proofs.

Figure legends should not exceed 300 words and ideally should be shorter. The figure is described first, then, briefly, the method. Reference to a method published elsewhere is preferable to a full description. Methods are not described in the text.

References are numbered sequentially as they appear in the text, followed by those in tables and finally by those in figure legends. Only papers published or in the press are numbered and included in the reference list. All other forms of reference should be cited in the text as a personal communication, manuscript submitted or in preparation. Text is not included in reference lists. References are abbreviated according to the *World List of Scientific Periodicals* (Butterworths, London, 1963–65). The first and last page numbers are included; reference to books includes publisher, place and date.

Abbreviations, symbols, units and Greek letters should be identified the first time they are used. Acronyms should be avoided whenever possible and, if used, defined. Footnotes are not used in the text.

Acknowledgements are brief and appear after the reference list; grant and contribution numbers are not allowed.

Supplementary information is material relevant to Articles or Letters which cannot, for lack of space, be published in full, but which is available from *Nature* on request.

Submission. Manuscripts can be sent to the Editor at 4 Little Essex Street, London WC2R 3LF, UK or at 1234 National Press Building, Washington, DC 20045, USA. A telephone and fax number should be included. Manuscripts or proofs sent by air courier to London should be declared as 'manuscripts' and 'value \$5' to prevent the imposition of import duty and value-added tax.